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# *ENZYMATIC FIXATION OF CARBON DIOXIDE IN α-KETOGLUTARIC ACID**

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During investigations on the heterotrophic assimilation of $CO_2$ by *Escherichia coli* and *Aerobacter aerogenes*, it was shown that the $C_5$ compounds, α-ketoglutaric acid and its precursor, glutamic acid, were more effective than the other members of the Krebs cycle or their precursors in replacing $CO_2$ in the metabolic requirements of the organisms.[1] It was proposed that a fixation over and above the Wood and Werkman reaction takes place and that this reaction may be of great importance to the cell. The present purpose is to report the enzymatic exchange of $C^{13}O_2$ with the carboxyl group of α-ketoglutaric acid and to show a new type of heterotrophic assimilation of $CO_2$ involving a $C_4$ and $C_1$ addition.

A cell-free enzyme preparation from *E. coli* decarboxylates α-ketoglutarate to succinate and $CO_2$ in the presence of malonic acid as an inhibitor of succinate oxidation. Under proper conditions the same preparation fixes $C^{13}O_2$ in the carboxyl group adjacent to the carbonyl carbon of the $C_5$ acid.

Any exchange reaction involving $CO_2$ and resulting in the formation of a carbon-to-carbon linkage is considered to be a fixation reaction. Attempts to demonstrate the carboxylation of succinate have thus far failed, probably because the equilibrium of the reaction is far to the side of decarboxylation and the quantities of α-ketoglutarate formed are too small to detect. A phosphorylated form of succinate is apparently involved since adenosine triphosphate enhances the fixation. The reaction as studied in the presence of malonate is a reversible oxidative decarboxylation involving a half mole of oxygen uptake for each mole of α-ketoglutaric acid utilized for the production of a mole of succinate and a mole of $CO_2$. Thus,

$$COOHCH_2CH_2CQCOOH + O \rightleftharpoons COOHCH_2CH_2COOH + CO_2.$$

*Methods.*—*E. coli* was grown for 16–18 hrs. at 30°C. in a medium con-

taining 0.8% glucose, 0.4% $(NH_4)_2SO_4$, 0.8% $KH_2PO_4$, 0.2% yeast extract and 10% tap water at an initial pH of 7.0. The cells were then harvested and ground with powdered glass and subsequently extracted with phosphate buffer solution (Kalnitsky, *et al.*[2]).

The exchange reactions were carried out in 125 ml. Warburg-Barcroft reaction vessels with two side arms. The final concentration of the $\alpha$-ketoglutarate was 0.01 *M* and that of malonate 0.05 *M*. Sodium salts of both acids were used. Depending on the activity of the juice, a quantity varying between 5 and 10 ml. was added to each flask. The mixture was buffered with 0.2 *M* phosphate at pH 6.6 and 0.07 *M* $NaHC^{13}O_3$. Appropriate concentrations of the two buffers were mixed with the substrate and enzyme after temperature equilibration (30.4°C.) had been established. pH of the resulting mixture was approximately 7.2 and the volume 25 or 30 ml.

The reaction was allowed to continue until approximately half of the substrate was utilized. The residual $CO_2$ in the mixture was then determined by aeration. Addition of the necessary reagents was made during the course of aeration and heating. The $C^{13}O_2$ was liberated by the addition of 6 *N* $H_2SO_4$ and collected in 8 ml. of 4 *N* carbonate-free NaOH in specially constructed carbon dioxide absorption tubes.

Since both malonate and $\alpha$-ketoglutarate are decarboxylated by the same reagents, the former was removed before decarboxylating the keto acid by the addition of 10 ml. of 0.025 *M* sodium bisulfite to the reaction mixture from which the residual $CO_2$ had been removed. The deproteinated sample was extracted for 72 hrs. with ether. The ether extract contained all of the original malonate as subsequently determined, plus the other dicarboxylic acid (succinic) formed during the reaction.

To the extracted solution was added 0.75 mM. of $NaHC^{12}O_3$, further acidified with $H_2SO_4$, heated and aerated for 15 minutes. The use of $C^{12}O_2$ rinse precluded residual $C^{13}O_2$ remaining to interfere with the isotope determination of the carboxyl carbon. The $C^{13}O_2$ content of the rinse was always determined.

$\alpha$-Ketoglutaric acid was degraded with ceric sulfate and $KMnO_4$ to succinate and $CO_2$. The $CO_2$ originates from the carboxyl adjacent to the carbonyl group; it was collected and the $C^{13}$ content determined on the mass spectrometer.

*Experimental.*—Succinic acid and carbon dioxide are the products formed by the aerobic dissimilation of $\alpha$-ketoglutaric acid in the presence of malonate by the cell-free enzyme preparation of *E. coli* (table 1). The reaction is similar to that reported by Ochoa[3] using cat heart as the source of $\alpha$-ketoglutaric dehydrogenase. When the action of the bacterial succinic dehydrogenase is blocked by sodium malonate, one molecule of succinate and one molecule of $CO_2$ are formed for each atom of oxygen taken

up in the oxidative decarboxylation of the keto acid. Succinate determinations by the silver salt method or by the oxidation with succinic dehydrogenase obtained from beef heart agreed well with the values for oxygen uptake and $CO_2$ evolved. No attempts were made to determine the products when malonate was omitted since the respiratory quotient of 1.2 to 1.3 obtained (theoretical 1.25) indicated a complete oxidation of $\alpha$-ketoglutarate to $CO_2$ and water.

TABLE 1

OXIDATIVE DECARBOXYLATION OF $\alpha$-KETOGLUTARATE BY A CELL-FREE ENZYME PREPARATION OF *E. coli*

| MATERIAL DETERMINED | WITH MALONATE* | R.Q. THEORY | R.Q. EXPTL. | WITHOUT MALONATE† | R.Q. THEORY | R.Q. EXPTL. |
|---|---|---|---|---|---|---|
| $\alpha$-Ketoglutarate utilized; mM. | 0.02 | ... | ... | 0.002 | .. | .. |
| Succinic acid formed; mM. | 0.017 | ... | ... | 0.0 | .. | .. |
| $O_2$ uptake; $\mu$l. | 109 | 2.0 | 2.1 | 103 | 1.25 | 1.30 |
| $CO_2$ liberated; $\mu$l. | 239 | | | 135 | | |

Total volume of reactants 2.8 ml. 0.0035 *M* $\alpha$-ketoglutarate, 0.05 *M* $PO_4$ buffer, pH 6.8, 1 ml. bacterial juice per cup. Temp., 30.4°C.

All values are corrected for enzyme blanks.

* 0.031 *M* sodium malonate.

† Time, 2 hrs.

The cell-free enzyme preparations are generally not highly active on $\alpha$-ketoglutarate or succinate but were used because malonic acid does not block the oxidation of succinate when the intact bacterial cell is employed (table 2). It was assumed that malonate did not penetrate the cell, therefore the bacteria were treated with various solvents to increase the permeability of the cell wall to the inhibitor. However, no satisfactory results were obtained. Quastel and Wooldridge[4] have shown that exposure of *E. coli* to toluene brings about a number of inactivations. The enzymes for lactic, succinic and formic acids were found intact. Our results are in agreement. Toluene and acetone treated cells remain active on succinate and become permeable to malonate; however, the same cells show no activity on $\alpha$-ketoglutarate. *n*-Propyl alcohol destroys both enzymes.

Recently, Klotz and Tietze[5] reported on the inhibition of succinic acid oxidation by structurally related sulfonic acids using rat liver homogenates. No such inhibition was noted with bacterial cells (table 3). However, in the case of bacterial juices, high concentrations are effective. The endogenous activity of the juice remains essentially unchanged even when a concentration as 0.08 *M* of inhibitor is used. Malonate not only inhibits succinic dehydrogenase but reduces the endogenous activity as well. Since toluene-treated cells remain active on succinic acid, this treatment does

TABLE 2

EFFECT OF MALONIC ACID ON SUCCINIC DEHYDROGENASE

| | METHOD OF INVESTIGATION | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MANOMETRIC; OXYGEN UPTAKE, μL. SUBSTRATES | | | | | THUNBERG; REDUCTION OF M.B. % REDUCTION SUBSTRATES | | |
| ENZYME PREPARATION | SUCCINATE | SUCCINATE + MALONATE* | α-KETOGLUTARATE | α-KETOGLUTARATE + MALONATE | MALONATE | TIME, HRS. | SUCCINATE | SUCCINATE + MALONATE |
| Suspension of washed *E. coli* | 446 | 516 | 186 | 193 | 30 | 2 | 90 | 90 |
| Toluene-treated suspension of *E. coli* | 103 | 3 | 18 | −10 | 0 | 2 | 80 | 0 |
| Acetone-treated suspension of *E. coli* | 201 | 63 | 62 | 33 | ... | ... | .. | .. |
| *n*-Propyl alcohol-treated suspension of *E. coli* | −6 | −2 | 23 | 6 | 0 | ... | .. | .. |
| Cell-free enzyme preparation of *E. coli* | 93 | 65 | 257 | 153 | −6 | 0.4 | 90 | 0 |

Manometric data: Total volume of reactants 2.3 ml. 0.0043 *M* α-ketoglutarate and succinate, respectively, 0.05 *M* $PO_4$ buffer, pH 7.0. Bacterial suspensions were added at the rate of 30 mg. per cup, dry weight, bacterial juice, 1 ml. per cup. Temp., 30.4°C.

Thunberg data: Total volume 6.0 ml. 0.0033 *M* succinate, 0.0066 *M* malonate, 0.066 *M* $PO_4$ buffer, pH 7.2. Bacterial suspensions, 0.5 ml. 10% suspension wet weight. Dilution of methylene blue 1/5000.

* 0.0086 *M* sodium malonate.

TABLE 3

OXIDATION OF SUCCINIC ACID IN THE PRESENCE OF 1,2-ETHANE DISULFONIC ACID

| ENZYME PREPARATION | SUBSTRATE | OXYGEN UPTAKE, μL. | METHYLENE BLUE REDUCTION TIME, HRS. | % REDUCTION |
|---|---|---|---|---|
| Suspension of washed *E. coli* | Succinate | 312 | 0.6 | 75 |
| | | | 0.4 | 95 |
| | Succinate + inhibitor | 352 | 0.3 | 95 |
| Toluene-treated *E. coli* | Succinate | 107 | 2 | 75 |
| | Succinate + inhibitor | 103 | 2 | 80 |
| Cell-free enzyme preparation of *E. coli* | Succinate | 224 | ... | .. |
| | Succinate + inhibitor | 60 | ... | .. |

Manometric data: Total volume 2.3 ml. 0.0043 *M* succinate, 0.05 *M* $PO_4$, buffer, pH 7.2, 0.08 *M* 1,2-ethane disulfonic acid. Bacterial suspensions were added at the rate of 30 mg. per cup dry weight, bacterial juice—1 cc. per cup. Temp., 30.4°C.

Thunberg data: Total volume 6.0 ml. 0.0033 *M* succinate, 0.16 *M* inhibitor, 0.066 *M* $PO_4$ buffer, pH 7.0. Bacterial suspensions, 0.5 cc. of a 10% suspension wet weight. Dilution of methylene blue 1/5000.

not alter permeability to 1,2-ethanedisulfonic acid. Type of the inhibition is yet to be determined. Apparently the sulfonate ion forms an enzyme complex of almost the same strength as that with malonate.

Because of the difficulties with intact cells, it was necessary to employ a cell-free preparation which not only oxidatively decarboxylates α-ketoglutarate to succinate and $CO_2$ but under the proper experimental conditions carboxylates succinate to form the $C_5$ keto acid (table 4).

TABLE 4

EXCHANGE OF HEAVY CARBON DIOXIDE IN THE CARBOXYL GROUP OF α-KETOGLUTARIC ACID DURING ENZYMATIC DECARBOXYLATION

| | | α-COOH OF α-KETOGLUTARATE | | | RESIDUAL $NaHC^{13}O_3$ —RINSE— | | | MALONATE | | COOH GROUPS |
|---|---|---|---|---|---|---|---|---|---|---|
| EXPT. NO. | ATP ADDED, mg. | α-KETOGLUTARATE ADDED, mM. | RECOVERED, mM. | EXCESS $C^{13}$ % | $C^{13}$ FIXED, mM. × $10^4$ | EXCESS $C^{13}$, % | RECOVERED,* mM. | EXCESS $C^{13}$, % | ADDED, mM. | RECOVERED, mM. | EXCESS $C^{13}$, % |
| 1 | .. | 0.25 | 0.16 | 0.171 | 2.7 | 2.79 | 0.75 | 0.00 | 1.25 | 1.24 | 0.00 |
| 2 | .. | 0.25 | 0.15 | 0.00 | 0.0 | .. | .. | .. | 1.25 | .. | .. |
| 3 | .. | 0.25 | 0.09 | 0.06 | 0.5 | .. | .. | .. | Not added | .. | .. |
| 4a† | .. | 0.25 | 0.18 | 0.102 | 1.8 | .. | .. | .. | 1.25 | 1.23 | .. |
| 4b | 40 | 0.25 | 0.19 | 0.372 | 7.0 | .. | .. | .. | 1.25 | 1.25 | .. |

Reactions were carried on in 125-ml. Erlenmeyer flasks with two side arms which were attached to the Warburg-Barcroft manometers. The final concentration of the α-ketoglutarate was 0.01 *M* and that of malonate 0.05 *M*. Depending on the activity of the juice, a quantity varying between 5 and 10 ml. was added to each flask. The mixture was buffered with 0.2 *M* phosphate, pH 6.6 and 0.07 *M* $NaHC^{13}O_3$ (3.113% excess). Appropriate concentrations of the two buffers were mixed with the substrate and enzyme after temperature equilibrium (30.4°C.) had been reached. The pH of the resulting mixture was approximately 7.2 and the volume 25 or 30 ml.

* mM. represent amounts recovered after treatment with $NaHC^{13}O_3$.

† Experiments 4*a* and 4*b* were carried out simultaneously.

It is apparent that the decarboxylation of α-ketoglutaric acid is reversible. The per cent excess of $C^{13}$ in the α-carboxyl of the keto acid was much higher when the reaction was conducted in the presence of malonate and adenosine triphosphate. In the absence of malonate the products of the reaction were water and $CO_2$. As soon as succinate was formed, it was oxidized. No detectable carboxylation of succinate occurred, consequently the reaction is not recognized as reversible. Since the error of the mass spectrometer is ±0.02, a per cent excess of 0.06% $C^{13}$ is questionable.

In the presence of malonate succinic acid accumulates to enhance the fixation of $CO_2$. At approximately half time of the reaction carboxylation is readily detectable.

In common with other carboxylations, this one is endergonic as is borne out by its enhancement by adenosine triphosphate (table 4).

It should be pointed out that at times reversibility could not be demonstrated. This may have been due to faulty procedure in the preparation of the juice. For example, when the juice is not centrifuged long enough to remove all of the unground cells, enough of them will be present to oxidize much of the succinate formed since their succinic dehydrogenase is not blocked by malonate, and consequently no $CO_2$ can be shown to be fixed. Partial denaturation of the enzyme or some component of the enzyme system may also account for experiment 2 in table 4. However, the discrepancies in this experiment are not too serious when compared with the overwhelming evidence for the reversibility (Expts. 1, 3, 4*a* and 4*b*). Any significant failure of malonate to block succinate dehydrogenation may result in fixation of carbon dioxide in the $\alpha$-ketoglutaric acid. Our previous experience would lead us to believe that this has not occurred under the conditions.

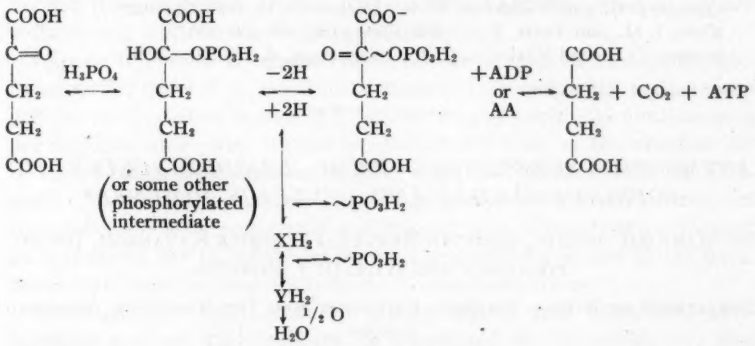
*Discussion.*—Although the present status of the Krebs cycle in bacterial metabolism is still uncertain, the evidence thus far obtained favors the occurrence of such a cycle in certain bacteria at least in principle. The results of Ajl, *et al.*,[1] on the replacement of $CO_2$ are of particular significance in this connection since metabolites normally occurring in that cycle are able to replace $CO_2$ thus showing not only a common function of these compounds in replacing $CO_2$, but potential interconversions among these compounds as well.

Every reaction of the Krebs cycle except the one studied has been shown to be reversible. According to the present findings, a complete reversal of the oxidative degradation of foodstuff would now be possible. By carboxylation and reduction, $\alpha$-ketoglutaric acid would be converted to citric acid and the latter would split into acetic and oxalacetic acids as shown by Brewer and Werkman.[6] Further, oxalacetic acid would be reduced to succinic acid by way of malic and fumaric acids, and the succinate thus formed would be converted to $\alpha$-ketoglutaric acid by reductive carboxylation. In this way, $\alpha$-ketoglutaric acid would be constantly regenerated. Therefore, $CO_2$ and $H_2$ entering the cycle at various points during metabolism would emerge as pyruvate which could then be used for the synthesis of carbohydrates.

In a system devoid of $CO_2$, $\alpha$-ketoglutaric acid may function in one or two ways. First, it may be oxidatively decarboxylated to yield succinic acid and $CO_2$, the former being utilized by the organism in place of $CO_2$ and secondly, the keto acid may function as a substrate for transamination, a mechanism responsible for the interconversion of proteins and carbohydrates. Since growth depends on the presence of carbon dioxide, which is replaced by $\alpha$-ketoglutarate to a greater extent than any of the $C_4$ acids,

it is entirely possible that during the normal metabolic processes of the bacterial cell the $C_1$ to $C_4$ addition may be of greater importance to the cell than some of the other fixation reactions known to occur.

The mechanism of the reversibility may be represented:




where X and Y are intermediary hydrogen acceptors.

Of the 50 energy-rich phosphate bonds that are generated when one molecule of glucose is completely oxidized, not more than two are formed during the initial anaerobic stages that lead to the formation of lactic acid. The residual 48 bonds therefore must arise during the subsequent oxidative stages. Twenty-four (~) must be generated from each molecule of lactate. Lactate is dehydrogenated to pyruvate and the keto acid oxidatively degraded through the Krebs cycle. But of all the reactions involved in this cycle only two are known to undergo oxidative decarboxylations yielding energy-rich phosphate bonds, i.e., the oxidative decarboxylation of pyruvate and of α-ketoglutarate. It follows that some 22 additional energy-rich phosphate bonds are formed as a result of other reactions. It is postulated that the additional bonds are formed during the transfer of electrons from the primary acceptor to oxygen. If such bonds are generated, their energy could conceivably be used to reverse the intermediary steps of the reaction studied. That this may actually be the case is borne out by the fact that ATP enhances the reversibility (table 4) by increasing the concentration of energy-rich phosphate bonds.

*Summary.*—Reversibility of the following reaction has been demonstrated with a cell-free enzyme preparation of *E. coli*.

$$COOHCH_2CH_2COCOOH + O \longleftrightarrow COOHCH_2CH_2COOH + CO_2.$$

Adenosine triphosphate enhances the reversibility.

Occurrence of this reaction explains the function of α-ketoglutaric acid in replacing $CO_2$.

* Journal Paper No. J-1578 of the Iowa Agricultural Experiment Station. Project No. 746.

[1] Ajl, S. J., White, A. G. C., and Werkman, C. H., *J. Bact.*, **54**, 23 (1947).

[2] Kalnitsky, G., Utter, M. F., and Werkman, C. H., *Ibid.*, **49**, 595–602 (1947).

[3] Ochoa, S., *J. Biol. Chem.*, **155**, 87–100 (1944).

[4] Quastel, J. H., and Wooldridge, W. R., *Biochem. J.*, **22**, 689–702 (1922).

[5] Klotz, I. M., and Tietze, F., *J. Biol. Chem.*, **168**, 399–400 (1947).

[6] Brewer, C. R., and Werkman, C. H., *Enzymologia*, **6**, 273–281 (1939).

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## *ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES III. COPRINUS SIMILIS AND LENTINUS DEGENER**

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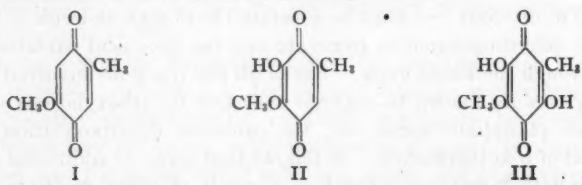
In the course of our investigations of the production of antibiotic substances by Basidiomycetes, two fungi, *Coprinus similis* and *Lentinus degener*,[1] were found to form the same antibacterial substance. This has been determined to be 5-methoxy-*p*-toluquinone (I).




The identification of this compound as a mold product is of particular interest because two related compounds, fumigatin (II) and spinulosin (III), have previously been obtained from the culture liquids of fungi. Fumigatin is produced by *Aspergillus fumigatus*[2] spinulosin by *Penicillium spinulosin*[3] and by *A. fumigatus*.[4]

*Antibacterial Activity of Culture Liquids.*—Each of the two fungi was grown at 25°C. in 2800-ml. Fernbach flasks containing 1 liter of modified Czapek-Doz medium with dextrose and corn steep solids on coils of beechwood shavings[5] which furnished mechanical support for the mycelium.

Cultivated under these conditions, *C. similis* produced in about one month culture liquids with an activity of 128 or 256 dilution units per milliliter when tested against *Staphylococcus aureus* by serial dilution. The antibacterial activity remained essentially unchanged in such cultures for two months or more. When the activity of the culture liquids reached

128 or 256 dilution units, the old culture liquid was decanted and replaced with fresh sterile corn steep medium. Within seven to ten days, sufficient antibacterial activity had developed to justify further reflooding. Mats sixty-one days old were successfully reflooded and some mats were reflooded three successive times. The maximum activity observed for our cultures was 256 dilution units per milliliter.

Culture liquids of *L. degener* developed an activity 2 to 4 times that observed for those of *C. similis*. In three to four weeks, the antibacterial activity of the culture liquids of *L. degener* was, as a rule, 256 dilution units per milliliter which with further incubation of a week or two reached 500 or 1000 dilution units and, on occasion, even 2000 dilution units per milliliter. If mats which produced culture liquids of activity such as mentioned were reflooded with fresh sterile medium, the new culture liquid evidenced an activity of 256 to 1000 dilution units per milliliter in five to ten days. Some mats were successfully reflooded 3 successive times.

Although both fungi produced culture liquids with substantial antibacterial potency, their activity as determined by the streak and disc methods was unsatisfactory. In fact, if the results from the streak and disc methods had been used as a standard of judgment, neither fungus might have been considered worthy of further investigation.[6]

Preliminary tests with the culture liquids of *C. similis* indicated that the antibacterial substance was largely destroyed by boiling at pH 8.8 or 11.0, and was destroyed in part by boiling at pH 4.1. These facts were borne in mind in efforts to isolate the material.

*Isolation of Crystalline Material.*—It was found that the antibacterial material could be removed almost quantitatively by extracting the culture liquid with ethyl ether or chloroform. From the ether or chloroform extracts a golden-yellow crystalline material was obtained either as plates or clusters of short prisms. These crystals had a characteristic aromatic odor which could be detected from the culture liquids also.

A procedure developed for isolating the crystalline material consisted of adjusting the pH of the culture liquid which was originally between 3.5 and 5.0 to 5.5 or 6.0, and extracting three times with one-fifth the volume of chloroform each time. The chloroform was removed under reduced pressure, and the crystalline residue was recrystallized from 95 per cent alcohol with addition of charcoal. The final product was dried *in vacuo* and stored in evacuated vials in the refrigerator to avoid possible oxidation.

In the following table, the results of the extraction of the culture fluid from each fungus is given:

| FUNGUS | AV. SIZE BATCH, LITERS | NO. OF BATCHES | AV. ACTIVITY, DILUTION UNITS PER ML. OF ORIGINAL CULTURE FLUID | | | ISOLATED, G. PER LITER CULTURE FLUID |
|---|---|---|---|---|---|---|
| | | | CULTURE LIQUID | CRUDE CRYSTALS | PURIFIED CRYSTALS | |
| *C. similis* | 8.5 | 2 | 128 | 192 | 303 | 0.12 |
| *L. degener* | 15.0 | 11 | 1005 | 1150 | 1060 | 0.37 |

For example, two batches of culture liquid from *C. similis* were worked up. They averaged 8.5 liters per batch and the culture liquid had an average activity of 128 dilution units per milliliter. The total antibacterial activity of the crude crystals was equivalent to 192 dilution units per milliliter of the original culture liquid, indicating complete or nearly complete extraction. The total activity of the purified crystals was equivalent to 303 dilution units per milliliter of culture liquid, which is within the probable error of the serial dilution method used in determining activity. The purified crystals recovered amounted to 0.12 g. per liter of culture fluid.

*Identity of Crystals from Two Fungi.*—The identity of the yellow crystalline material from the culture liquid of *L. degener* and that from the culture liquid of *C. similis* was established by mixed melting points of purified samples of crystals obtained from the two sources, by carbon and hydrogen analyses of both samples and by a comparison of the bacterial spectra of crystalline material obtained from culture liquids of each fungus. No evidence for the production of more than one antibacterial substance by either fungus was observed.

*Bacterial Spectrum.*—The inhibitory concentration at twenty-four hours in micrograms per milliliter as found in three determinations for crystals obtained from the culture fluid of *C. similis* and from that of *L. degener*, was as follows. Tests of the same number were run simultaneously. The crystals were dissolved in 10, 15 or 20 per cent alcohol, and the determinations were made by serial dilution.

| ORGANISM | MINIMUM INHIBITORY CONCENTRATIONS IN μG. PER ML. CRYSTALS FROM *C. similis* | | | CRYSTALS FROM *L. degener* | | |
|---|---|---|---|---|---|---|
| | (1) | (2) | (3) | (1) | (2) | (3) |
| *Bacillus mycoides* | 32 | ... | 16 | 64 | ... | 16 |
| *Bacillus subtilis* | 2 | 2 | 4 | 4 | 2 | 4 |
| *Escherichia coli* | 64 | 64 | 64 | 128 | 64 | 32 |
| *Klebsiella pneumoniae* | 16 | 64 | 32 | 64 | 64 | 16 |
| *Pseudomonas aeruginosa* | 256 | 128 | 256 | 256 | 128 | 256 |
| *Mycobacterium smegma* | 128 | 64 | 128 | 128 | 128 | 64 |
| *Staph. aureus* | 1.0 | 0.5 | 1.0 | 0.5 | 0.5 | 0.5 |

*Antifungal Action.*—The effect of crystals prepared from the culture liquids of *C. similis* and *L. degener* on the inhibition of several fungi was determined. A spore suspension of each fungus was tested by serial dilution in 1-ml. quantities of a peptone medium[7] at pH 6.0. *Trichophyton mentagrophytes* was incubated at 30°C.; the other fungi at 25°C. The activity of both samples of crystals was essentially the same. *T. mentagrophytes* was the most sensitive; *Gliomastix convoluta*, *Stemphylium consortiale* and *Penicillium notatum*, the most resistant.

| | MINIMUM INHIBITORY CONCENTRATION[8] IN μG. PER ML. AT END OF | | |
|---|---|---|---|
| | 24 HRS. | 42 HRS. | 72 HRS. |
| *Staph. aureus* | 1 (1) | 1 (1) | .... |
| *A. niger* | 16 | 32 | 32 |
| *Chaetomium globosum* (USDA 1042.4) | .... | 16 (16) | 16 (32) |
| *Gl. convoluta* (PQMD 4c) | 64 (64) | 128 (128) | 128 (128) |
| *Memnoniella echinata* (PQMD 1c) | .... | 32 (32) | 32 (64) |
| *Myrothecium verrucaria* (USDA 1334.2) | .... | 64 (64) | 64 (64) |
| *St. consortiale* (PQMD 41b) | .... | 64 (128) | 128 (128) |
| *P. notatum* | 128 (128) | 128 (128) | 128 (128) |
| *Phycomyces blakesleeanus* (+) | 32 | 32 | 64 |
| *T. mentagrophytes* | .... | 4 (4) | 8 (8) |

*Identification of Crystalline Material.*—The identification of the crystalline material as 5-methoxy-*p*-toluquinone was based on the following evidence.

1. The substance showed typical quinone behavior: its solution in concentrated sulfuric acid was bright orange-red; an alkaline solution darkened rapidly to a purplish brown color and the compound could not be recovered on acidification; it reacted with carbonyl reagents; it took up bromine in carbon tetrachloride; it yielded a purple crystalline derivative with aniline; it gave a blue-violet color changing to blue-green and finally reddish-brown on treatment with ethyl cyanoacetate and ammoniacal ethanol.[9]

2. The melting point was in agreement. Luff, Perkin and Robinson[10] report a melting point of 170–172°C. with previous softening at 165° for (I).

3. Analyses established an empirical formula of $C_8H_8O_3$.

4. The compound underwent the same reactions as those reported for (I) and gave products with the reported melting points

(*a*) On reduction with sulfur dioxide, a white solid was obtained which crystallized from water in long, fine needles of melting point 122–124°C. The melting point reported for the reduction product of (I) is 124°C.[10]

(*b*) "Thiele acetylation" yielded a white solid which crystallized in prismatic needles of melting point 91–92°C. Erdtman[11] reports 91–92°C. as the melting point for 4-methoxy-2,3,5-triacetoxytoluene obtained by this method from (I).

(*c*) Treatment with acetylchloride and acetic acid gave a white solid which crystallized from acetic acid in fine needles of melting point 126–127°C. The melting point of 3-chloro-2,5-diacetoxy-4-methoxytoluene obtained from (I) by this method is 126°C.[11]

5. The positions and heights of the maxima in the absorption spectrum are in agreement with those reported for (I).[12]

Oxford[13] compared the antibacterial activity of a number of derivatives of toluquinone and benzoquinone. He found 5-methoxy-*p*-toluquinone[14] to be one of the more active compounds effective on gram-positive organ-

isms but without striking action on gram-negative bacteria. It was more active than fumigatin which in turn had a greater antibacterial effect than spinulosin. The inhibitory concentrations reported by Oxford for *Staph. aureus* are one-third of those we found for this compound or less. This is probably because of differences in strains of bacteria and differences in methods of test.

Some of its characteristics militate against the possible therapeutic application of 5-methoxy-*p*-toluquinone. Its solubility in water or saline was low (0.5 mg. per milliliter). When exposed to 5 per cent blood for three hours at 37°C., its activity was reduced on the average by 50 per cent. A few preliminary tests indicated that 25 mg. per kilogram killed one-half the mice injected intravenously within five days.

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[1] We are indebted to Dr. José Emilio Santos Pinto Lopes, Instituto Botanico, Lisbon, Portugal, for the culture of *C. similis*, and to Professor Roger Heim, Museum National d'Histoire Naturelle, Paris, for that of *L. degener*.

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[7] This medium contained per liter 1.5 g. $KH_2PO_4$, 0.5 g. $MgSO_4.7H_2O$, 50 g. dextrose, 2 g. neopeptone, 1500 mμ moles of thiamine, and a mixture of minor mineral elements as used in this laboratory.

[8] The figures in parentheses are the values for crystals from *L. degener;* the others are those for crystals from *C. similis*. We are indebted to Dr. W. L. White for cultures of the fungi with USDA or PQMD numbers.

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[14] According to the system of nomenclature used by Oxford, this compound is 4-methoxy-*p*-toluquinone.

# THE BEARING OF THE LIVING METASEQUOIA ON PROBLEMS OF TERTIARY PALEOBOTANY

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The discovery of huge living trees in California in 1769, followed by the naming and description of the coast redwood, *Sequoia sempervirens*, by Endlicher in 1847,[1] served as a prelude to the recognition of fossil redwoods in many parts of the northern hemisphere. Leafy shoots from the Oligocene of France, originally assigned to *Taxites langsdorfii* by Brongniart[2] were correctly transferred to the genus Sequoia by Heer in 1855.[3] Heer also identified as members of this genus specimens sent to him from the collections of polar explorers, from Tertiary deposits in Greenland, Iceland, Spitzbergen, Siberia, Sachalin, northern Canada and Grinnell Land.[4] Bringing to North America much of the tradition of European paleobotany, Lesquereux recognized several species of fossil Sequoia in the western United States, including the widely distributed *S. langsdorfii*.[5] During ensuing years the occurrence of Sequoia has been widely noted over the northern hemisphere, at middle latitudes in rocks assigned to middle Tertiary, and at high latitudes in rocks of older Tertiary and Cretaceous age. From the pattern of its occurrence during these later periods of earth history, the paths of its migration southward have been charted.[6, 7] Its environment in past ages has been reconstructed from comparisons with the modern habitat of the coast redwood in California and Oregon.

A fossil cone described as *S. heerii* by Lesquereux[8] from beds of Oligocene age on Medicine Lodge Creek (Sage Creek), Montana, differs from other American cones referred to Sequoia in its attachment on a "naked pedicel." Lesquereux believed that the absence of needles on this "pedicel" (if it had had needles it could not have been properly so designated) had resulted from maceration. In later years identical stalked cones have been found at other Tertiary localities in western North America, such as Bridge Creek, Oregon, and Elko, Nevada; some of the cones figured by Heer as *S. nordenskioldi* from the Eocene of Spitzbergen,[9] and as *S. langsdorfii*[10] and *S. brevifolia*[11] from the Eocene of Greenland are also borne on stems lacking needles. For some time it has been apparent that this type of Sequoia cone is readily distinguishable from those of the living species and of other American fossil species; one of my students has even suggested that there are differences sufficient to justify establishing a new genus.[12]

In 1936 Endo[13] pointed out for the first time a significant character in a

cone of this type, assigned to *S. japonica* from later Tertiary deposits of Japan and Korea. His description and remarks are as follows:

"Description: Cone rather small, spherical, ca. 16 mm. in diameter; cone-scales ca. 16 in number, arranged in 4 longitudinal rows, each row with 4 scales; escutcheon hexagonal, transversely grooved and radially wrinkled, 10 mm. wide, 3 mm. high. Peduncle stout, 2 mm. in diameter.

"Leaves with decurrent base, sessile, arranged in 2 lines; linear, bluntly mucronate at apex, 10 mm. long, 2 mm. wide; upper surface slightly furrowed along the midrib.

"Remarks: The arrangement of the cone-scales in the present species is in longitudinal rows, while being usually spiral in most other species. It has this characteristic feature in common with the cones from the Miocene of John Day valley, once described by Lesquereux as belonging to *Sequoia langsdorfii;* unfortunately the latter is too imperfect for farther comparison with the present materials."

Endo's use of the term "peduncle" is unfortunate, since the stems to which the cones are attached appear in at least two specimens (figures 6, 13) to bear leaves. They may best be considered leafy shoots bearing terminal cones. An obscure photograph (figure 12) of a vegetative shoot shows its needles in opposite position, although Endo does not mention this feature in his description of the leaves. In fact, many fossil shoots with oppositely placed needles had been figured as far back as the days of Heer with no reference to this readily observable character.

It remained for Miki[14] to found a new genus, Metasequoia, in 1941 on the basis of stalked cones and leafy shoots from Pliocene deposits at Osusawa and elsewhere in the clay beds of Central Hondo. His description and discussion (in altered sequence) are here quoted:

"Character: Cone pedunculate, scale decussate, shield-form; peduncle with distichous scars of leaves and scaly leaves at the base. Shoot deciduous; leaf distichous, linear, obtuse, petiolate; stomata parallel to the midrib.

"The remains have usually been referred to Sequoia or Taxodium, indeed the cone is like that of Sequoia and the foliage shoot is somewhat like those of Taxodium.

"The cones were never found connected to branches, but as the leaf-scars on the peduncle are also distichous, it is conceivable that the cones and the shoots belonged to the same plant. The foliaged shoots seem to be lateral branches shedding in autumn, because their length is usually constant and the proximal end is covered by scaly leaves, although they have no scaly bud on the top and the branches two or more years old have two or more bud scars on the nearly same point as in Fig. 8 Ge.

"The cone is distinguished from that of Sequoia by the decussate arrangement of scales, and by the delicate peduncle having scale leaves at the base. The foliaged shoot differs from Sequoia by distichous arrangement of leaves and by the brittle petiole. At a glance the shape of the shoot of fragmental remain seems to be Taxodium or Cephalotaxus but it differs from Taxodium by distichous leaf and parallel arrangement of stomata on it and from Cephalotaxus by short delicate shoot without scaly bud at the terminal and by the obtuse top of leaf.

"The decussate arrangement of cone-scales is not found in living Taxodiaceae, but a common character in Cupressaceae. The shedding of lateral foliaged shoot with linear leaves is common in Glyptostrobus and Taxodium. So it is sure that the remains

belong to Taxodiaceae but as the characters do not harmonize with those of the living forms, a new genus Metasequoia is established."

Since several of the cone-stems which he figures (*A—g, h, i, j, k*) show scaly leaves or leaf scars, it seems clear that they are modified leafy shoots rather than peduncles. Miki uses the term "distichous" to describe the position of needles on the foliage shoots; whether he meant to indicate their opposite position (and I am inclined to believe from his phrasing that he did) is uncertain, but in any event the term carries no such implication; Sargent[15] even defines "distichous" as "leaves arranged alternately in two vertical ranks," though the word may be equally well used for opposite position. The significant feature of Miki's description is his recognition of the association of cones bearing decussate scales with leafy shoots which were deciduous. He assigns two species to Metasequoia, of which one, *M. disticha*, was originally described as *S. disticha* by Heer[16] from the Eocene of Spitzbergen on the basis of leafy shoots. The second, *M. japonica*, is the species originally described by Endo as *S. japonica* from Miocene and Pliocene deposits in Japan and Korea; it is distinguished from *M. disticha* by the shape and fewer numbers of its cone-scales, distinctions which may not prove to be of specific significance in the light of our present knowledge of the genus. Miki's assignment of these fossil cones and shoots to a new genus, and his conclusion that even though they do not occur in direct attachment they are parts of the same deciduous plant, represent an outstanding discovery in Tertiary paleobotany. It is all the more remarkable, coming as it did only a few years before a second major discovery.

Miki's paper had probably not been read by any paleobotanist or botanist outside of Asia when it was announced, in the spring of 1946, that three trees of Metasequoia had been found living in Central China. Tsang Wang, attached to the Bureau of Forestry of the Ministry of Agriculture, had brought back to Nanking specimens collected at the village of Motao-chi, in eastern Szechuan, about 140 miles northeast of Chungking. This material was studied by W. C. Cheng, Professor of Forestry at National Central University, and H. H. Hu, Director of the Fan Memorial Institute of Biology, both of whom have a wide knowledge of the living plants of China. They soon realized that it represented no known living tree, and comparisons showed that the cones and leafy shoots were essentially the same as those described by Miki from fossil specimens in Japan. Additional collecting by C. J. Hsueh in 1946 resulted in the discovery of several additional trees, and greatly augmented the material available for study. During the fall of 1947, C. T. Hwa, also a student of Professor Cheng, spent several months in the region, with financial support provided by the Arnold Arboretum of Harvard University through the coöperation of E. D. Merrill,[17] and with a small grant from the University of California.

In the region to the south, in western Hupeh, Hwa found several hundred additional trees of Metasequoia, made a comprehensive collection of the woody plants of this general area, and brought out a large quantity of viable seeds. With adequate material for study at hand, Hu and Cheng have recently published an account of this new tree under the name *M. glyptostroboides*.[18] In an earlier paper, Hu[19] had mentioned the discovery of this living tree in his brief discussion of another fossil species, *S. chinensis*, which had been described by Endo from the Oligocene flora of Fushun, Manchuria;[20] Hu correctly transferred it to the genus Metasequoia on the basis of its stalked, decussate-scaled cones. The leaves and cones of the living trees of Metasequoia, as described by Hu and Cheng, differ in no essential respects from those of the fossils on which Miki based his generic description. But there are four additional characters, of particular interest to paleobotanists, which are mentioned by these authors: (1) "opposite, distichously arranged foliage shoots"; (2) "leaves—opposite"; (3) "staminate flowers axillary and terminal, opposite, on racemose or paniculate flowering branchlet-system"; (4) cone-bearing twigs "with decussate linear leaves before maturity, and with conspicuous leaf scars and with persistent bracts at the base in winter." This paper by Hu and Cheng, describing the occurrence and external characters of *M. glyptostroboides*, represents a major contribution to the botany of China and of the world.

With this significant information regarding Metasequoia, both fossil and living, coming over from Japan and China, I have reëxamined with graduate students at the University of California[21] the abundant conifer material in our Tertiary collections from the western United States, and later the collections at the United States National Museum. We have also studied the descriptions and illustrations of Heer and others, covering material from Cretaceous and Tertiary horizons elsewhere in the northern hemisphere. All of the specimens previously referred to *S. heerii*, and many of those assigned to *S. langsdorfii* and several other fossil species, have the cone or foliage characters of Metasequoia. In addition, staminate aments like those of the living species have been found in the Bridge Creek flora (Oligocene) of Oregon, and the Elko flora (Miocene) of Nevada, in association with typical cones and leafy twigs of Metasequoia. These fossil aments and twigs from Nevada had previously been referred to Taxodium.[22] It has become increasingly apparent that many of the specimens assigned to Sequoia and Taxodium from the Cretaceous and Tertiary floras of North America are properly referable to Metasequoia. A generic revision of some of these is now in preparation, under the auspices of the American Philosophical Society and the Carnegie Institution of Washington. At this time it seems desirable to rewrite the description of Metasequoia on the basis of the paleobotanical evidence. In this description

and the following discussion the data provided by fossil specimens will be the primary consideration, though in some cases our knowledge of corresponding parts of the living tree has enabled us better to interpret them. All consideration of fossil stems is here omitted, since study of the wood of Metasequoia, modern and fossil, has not progressed to a point where significant distinguishing characters between it and Sequoia have been noted.

*Genus Metasequoia Miki.—Description.*—Foliage shoots straight, decussate; alternate pairs which come out from top and bottom of branch are twisted approximately 90° into horizontal plane of laterally disposed pairs so that all lie along one plane on branch, in opposite pairs; pairs originating in vertical plane often missing and represented by scars preserved on top surface of branch; diverging at angles approaching 90°, commonly ranging in length up to 8 cm., deciduous. Needles decussate, twisted with their decurrent bases to form two oppositely paired ranks, closely spaced, diverging at angles approaching 90° in typical, mature specimens; up to 1.8 cm. long, averaging about 8 mm., longest on proximal half of shoot and gradually reduced in length toward its tip; up to 2 mm. in width; obtusely tipped, narrowed and twisted at point of attachment to decurrent base; midrib well defined, with stomata parallel to it on ventral surface. Staminate cones ovate, up to 5 mm. long, with decussate bracts; closely spaced, sessile and decussate on elongate spikes; rotated into one plane so that they appear to be attached in opposite pairs. Ovulate cones globose to ovoid or elliptic, up to 2 cm. long and 1.5 cm. wide, averaging 1.5 by 1.2 cm. (at one American locality (Mollala) they are globose, 1.6 to 2.4 cm. in diameter, open cones slightly broader); cone-scales 12 to 24, decussate, peltate on broad-based stalks, discs lenticular to hexagonal, with transverse, medial grooves. Seeds about 3 mm. long, 1 mm. wide, the surrounding wings asymmetrical, notched at the apex, with over-all length of 4 to 5 mm., width of 3 to 3.5 mm. Cones terminal on stout, straight or curved shoots; cone-bearing shoots probably lateral, up to 4 cm. long and 2 mm. in diameter, more slender at the middle, bearing scaly leaves at base in complete specimens, rarely with scaly leaves or leaf-scars preserved along the shoot, probably widely spaced and evidently decussate.

*Discussion.*—It is unusual to find leafy shoots attached to branches in the fossil record. Their position is decussate in every instance, though the shoots that come out at right angles to the plane of the branch are commonly missing, and may be represented only by scars which show on the exposed surface of the slab. These fossil shoots average somewhat longer than those of the living *M. glyptostroboides* which we have examined. Some specimens, especially those from Elko, Nevada, and Mollala, Oregon, both of Miocene age, have slender, curving shoots with needles more

openly spaced and directed distally at higher angles than the typical specimens from Bridge Creek and other localities; studies of foliage of living trees have not progressed to a point where we can determine whether there is corresponding variation in *M. glyptostroboides*. Differences of opinion have been expressed regarding the mode of attachment of the needles. I agree with Stebbins[23] that their position is decussate; this may be readily observed at the tips of young shoots on seedlings which we have growing in Berkeley. The older shoots on herbarium specimens, as well as the leafy shoots preserved as impressions on our fossils, also show clearly the alternating position of attachment of successive pairs of needles and the twisting of their decurrent bases to bring all the pairs of needles into a single plane. Thomas Morley, graduate student in botany at the University of California, has at my suggestion sectioned the stem of a leafy shoot of the living *M. glyptostroboides* (sheet No. 753369, Univ. Calif. Herbarium). He finds the leaf gaps opposite at each node, with each successive pair at right angles to the pair below. However, in alternating nodes the leaf gaps and the decurrent leaf bases are twisted approximately 90°, which brings all the points of attachment of the needles into essentially the same plane along the shoot. An original decussate arrangement is thus confirmed by Morley's slides. No difficulty has been experienced in recognizing the opposite position of the needles in well-preserved fossils of Metasequoia; under low magnification the relations of needles and leaf bases to the shoot are as clear as with modern material; both living and fossil specimens are distinguishable at a glance from leafy shoots of Sequoia and Taxodium in which the needles are alternately disposed along the shoots. Like the pairs of needles, the decussately attached staminate cones are twisted into a single plane along the twig so that they are disposed in opposite or nearly opposite pairs. The staminate cones of a fossil specimen from the Oligocene Bridge Creek flora of Oregon are much smaller than those from Elko, and appear to represent a less-developed stage; in the living species there is a similar range in size. A specimen from Elko shows six spikes, in close association like those of the living species. The considerable range in size and shape of the ovulate cones, and in the numbers of their cone-scales, is no wider than is to be found in cones of the living species, and we have no present basis for determining whether such variation will provide criteria for recognizing two or more species.

As stated in the description, needles are commonly lacking from the cone-bearing shoots, and even their attachment scars are difficult to make out on most fossil specimens. The scars are readily visible on shoots of the living plant, and needles may remain in attachment while the cone is on the tree. When it has fallen, the needles are shed; the fossil record is made up exclusively of cones which have fallen to the ground, and the presence of needles on their shoots is not to be expected. Since this dis-

cussion deals with fossil material, in which the cones and leafy shoots are not commonly attached to branches, we have had to qualify certain of our statements regarding their mode of attachment. However, there is little doubt that this follows the general pattern of the living *M. glyptostroboides*, in which the vegetative and reproductive shoots, and their needles and scales, are prevailingly decussate.

A survey of the characters of Metasequoia as seen in fossil material, and a comparison of these characters with those of Sequoia and Taxodium, provides the following bases for distinguishing this Chinese genus:

(1) Leafy shoots decussate, disposed distichously in opposite pairs along the branches in Metasequoia; spiral, disposed distichously and alternately in Sequoia and Taxodium.

(2) Leafy shoots deciduous in Metasequoia and Taxodium; remaining on the branches 3 to 4 years in Sequoia.

(3) Needles decussate on the shoots of Metasequoia, twisted and disposed distichously in opposite pairs; spirally disposed, commonly distichous, on the shoots of Sequoia and Taxodium.

(4) Stomata in parallel bands on each side of the midrib in Metasequoia and Sequoia; irregularly transverse in Taxodium.

(5) Staminate cones decussate on spikes, twisted into distichous, opposite pairs, in Metasequoia; spirally disposed on spikes in Taxodium; solitary in the axils of terminal needles in Sequoia.

(6) Pistillate cones terminal on elongate, probably lateral, leafy shoots in Metasequoia (they are lateral in the living species), the needles widely spaced, deciduous, leaving a naked cone-shoot on shedding; terminal on short scaly shoots which develop at the ends of leafy shoots of the preceding year in Sequoia; cones at the ends of branches in Taxodium.

(7) Cone scales decussate in Metasequoia; spirally disposed in Sequoia and Taxodium.

Our conclusion that Metasequoia rather than Sequoia was the dominant conifer of the Arcto-Tertiary Flora, whose southward migration from high latitudes has been so well established,[6, 7] calls for reconsideration of the paleoecology and floristics of the Tertiary period. It is of primary significance to note that it was not an evergreen but a deciduous conifer which ranged northward to latitude 82° in Grinnell Land during the Eocene. The deciduous habit of Metasequoia was wholly consistent with that of the majority of its angiosperm associates in the older Tertiary floras from high northern latitudes. This same group of trees, only slightly altered in composition, is widely known at middle latitudes, both in North America and Asia, during middle Tertiary time. The need for determining the associates of the living *M. glyptostroboides* becomes at once apparent, for they may represent a closer approach to the Arcto-Tertiary Flora than any modern vegetation as yet studied. It is necessary also to learn as much

as possible about the modern environment of Metasequoia, to serve as a guide for the reconstruction of its habitats in past ages.

In February and March, 1948, with Dr. Milton Silverman, Science Writer for the San Francisco *Chronicle*, I made a brief trip to western Szechuan and eastern Hupeh under the auspices of the Save-the-Redwoods League, and with the cordial coöperation of Chinese scientists and of Chinese and American officials. It was our good fortune to have accompanying us C. T. Hwa who had previously collected extensively in this area. The redwoods of China live in valley bottoms and slopes at altitudes from 4000 to 4450 feet. The discovery trees at Mo-tao-chi, Szechuan, including the largest tree observed[24] with a diameter of 64 inches above the buttress and a height of 98 feet, are growing in the midst of rice-paddies, and no other trees occur within 100 yards. But in the valley of Shui-hsa-pa, in western Hupeh, scores of Metasequoias were observed not only on the borders of rice-paddies on the floodplain, but extending up ravines under conditions which appeared to be relatively natural.[25] This is in no sense a forest, for trees occur largely in isolated ravines in association with other conifers and with deciduous hardwoods which show every evidence of being second growth. These associates had been widely noted at corresponding elevations during our 5-day trip into the area, and the plant formation has been described by Cheng[26] as occurring between 400 and 2000 meters elevation in this area. Among the more common hardwoods noted in immediate association with Metasequoia are chestnuts (*Castanea henryi* and two other species), a small-leafed oak (*Quercus glandulosa*), sweet gum (*Liquidambar formosana*), and cherry (Prunus) of an undetermined species. An evergreen shrub related to our spice-bush (Lindera) is one of the most abundant members of the understory. On adjacent higher slopes the birch (*Betula luminifera*) and the beech (*Fagus longipetiolata*) are common, and in one ravine there is a large tree of katsura (*Cercidiphyllum japonicum*, var. *chinensis*). Four evergreen conifers, *Cunninghamia lanceolata*, *Cephalotaxus fortunei*, *Pinus massoniana* and *Taxus chinensis*, are of regular occurrence here, and small fan palms (*Trachycarpus fortunei*) were noted at various places in the Shui-hsa-pa area. This assemblage is essentially the same as that recorded in the fossil record of the Eocene from high latitudes; many of the figured specimens of Metasequoia from Greenland and Alaska have on the same slabs leaf impressions of katsura or birch; the Oligocene and Miocene floras from the John Day Basin of Oregon and other localities in the western United States include all of the angiosperm genera (except the palm) above recorded, in association with fossils of Metasequoia. Numerous genera which are common members of the Arcto-Tertiary Flora, both in the Eocene of high latitudes, and in middle Tertiary deposits at middle latitudes, have not yet been noted in immediate association with Metasequoia

at Shui-hsa-pa; however, our field work was of limited duration and carried on at a season unfavorable for easy recognition of deciduous trees. We already know that species of Alnus, Acer, Carpinus, Ostrya and Ulmus have been observed within a few miles of Metasequoia trees, and it is probable that other genera known in the fossil record will be recorded during the field season of 1948 while Professor Cheng and his associates are working in this region.

Prior to the study of the trees of this region, the nearest surviving equivalents of the Arcto-Tertiary Flora which I have seen are the mixed bald cypress (Taxodium)—hardwood forest of the Wabash river valley in Illinois and Indiana, and the hardwood forests with associated conifers at middle altitudes on the Island of Hondo in Japan. The occurrence in the Shui-hsa-pa region of Metasequoia and Cercidiphyllum now restricted to Asia,[27] together with many angiosperm genera which occur today in North America as well, gives these groves in the ravines of western Hupeh a closer resemblance to the Eocene floras of Greenland, Spitzbergen and Alaska, and to the Oligocene-Miocene floras of Oregon and Manchuria than any living group of plants known to me. Not only has Metasequoia come down through the ages to survive in Central China, but the whole assemblage of which it is a part has had a long geologic history, and has participated in wide migrations. During these movements covering thousands of miles, and continuing for millions of years, some genera have disappeared and others have been added; minor changes in leaf or fruiting characters which are the basis for specific distinctions have appeared; but the Arcto-Tertiary Flora as we know it from early Tertiary rocks at high northern latitudes is so fully represented in the Metasequoia groves of Central China that there can be no question as to the holarctic origin of this modern vegetation.

That being the case, we may turn to this region for suggestions regarding the physical environment occupied by the Arcto-Tertiary Flora at many localities in the northern hemisphere during past ages. The nearest station which provides climatic data is Chungking, 140 miles to the southwest, and at an elevation more than 3000 feet lower. Annual precipitation here (43-year record) is 43.1 inches, of which 2.3 inches fall in the winter, 11.1 inches in the spring, 17.7 inches in the summer, and 12 inches in the autumn. The mean annual temperature (44-year record) is 66.2°F., ranging from a low of 48.6° in January to a high of 84.4° in August; the mean monthly minimum (6-year record) ranges from 35° in January to 70° in August, and the extreme lowest recorded temperature in 25 years is 28.9°; the mean monthly maximum (6-year record) ranges from 59° in January to 104° in August, and the extreme highest recorded temperature in 25 years is 111°. Relative humidity averages about 82% (16-year record), and there is little monthly variation. It seems probable that at

Shui-hsa-pa, 4000 feet in elevation, there is higher rainfall and a wider range in temperature. At the time of my visit in March, temperatures were mild and light rains fell daily. Villagers reported that there is no snow or frost, and the general aspect of the understory, made up of evergreen shrubs in the Lauraceae, Theaceae, Euphorbiaceae and Palmae, is suggestive of winter temperatures which rarely fall below freezing. Discussing the climate of the Red Basin of Szechuan, on the eastern rim of which our area is located, Cressey has stated:[28] "The climate is temperate and mild. Despite the location in the interior of the continent, it is protected from extremes of temperature by the surrounding mountains, and the contrast between summer and winter is not great. Summer temperatures seldom exceed 100°F., while during the winter the thermometer does not usually drop below freezing."

The only vegetation in North America which is closely comparable with Metasequoia and its associates of Central China is the bald cypress-hardwood forest of the south Atlantic coastal plain and the Gulf of Mexico,

TABLE 1

SHOWING PRECIPITATION AND TEMPERATURE IN REGIONS OCCUPIED BY METASEQUOIA, TAXODIUM AND SEQUOIA

| | ALTITUDE, FEET | PRECIPITATION, INCHES | | | | | FAHRENHEIT TEMPERATURE | | |
|---|---|---|---|---|---|---|---|---|---|
| | | WINTER | SPRING | SUMMER | AUTUMN | ANNUAL | MEAN ANNUAL | EXTREME MAX. | EXTREME MIN. |
| Chungking | 754 | 2.3 | 11.1 | 17.7 | 12.0 | 43.1 | 66.2 | 111 | 28.9 |
| Brunswick, Ga. | 14 | 9.3 | 9.8 | 18.6 | 12.0 | 49.7 | 68.4 | 103 | 13.0 |
| Glennville, Ga. | 175 | 9.7 | 9.9 | 17.4 | 9.9 | 46.9 | 67.1 | 106 | 11.0 |
| Hammond, La. | 44 | 14.9 | 14.0 | 18.1 | 11.2 | 58.5 | 67.5 | 106 | 1.0 |
| Eureka, Calif. | 62 | 19.5 | 10.2 | 1.0 | 8.3 | 39.0 | 51.6 | 85 | 20.0 |

extending northward up the Mississippi Valley to Illinois and Indiana. This is a lowland forest, reaching a maximum altitude of about 475 feet in the valley of the Wabash River. At latitudes corresponding to Shui-hsa-pa in Louisiana and Georgia, this forest lives within a few tens of feet of sea level, ranging up to about 200 feet in Georgia. As in Central China, precipitation is well distributed and adequate over all this region (table 1), with more than half falling in the spring and summer months. In the southeastern United States at the latitude of Shui-hsa-pa, temperatures show essentially the same mean average, but the extreme minima are much lower.

The bald cypress, *T. distichum*, of the American forest has its best development in swamps and on swamp borders, but its occurrence with hardwoods on moist floodplains more closely resembles the environment of Metasequoia in Central China. All of the angiosperm associates in the groves at Shui-hsa-pa have been noted with the exception of Cercidiphyllum and Trachycarpus which are confined to Asia; Sabal takes the

place of the latter genus in the southern United States. In spite of their geographic separation and marked topographic differences, the general aspect of the Taxodium-hardwood forest is surprisingly similar to that of the Metasequoia-hardwood assemblage. This resemblance is the more significant since during middle Tertiary time Taxodium and Metasequoia lived together in western North America and in northeastern Asia, in association with the same angiosperms; during early Tertiary time, these two conifers were widely distributed at high latitudes and with the same genera of hardwoods. Taxodium is almost as widely distributed in the Arcto-Tertiary Flora as Metasequoia. We conclude that this forest type had its origin at the north, in Eocene and Cretaceous time, in a region with a summer-wet and winter-cool climate; the deciduous habit of all the dominant trees, including Metasequoia and Taxodium, is consistent with such a climate. By Oligocene and Miocene time, a similar forest was widely established as far south as Oregon and Nevada, and down into Japan and Manchuria. By the end of the Miocene, Metasequoia appears to have disappeared from this continent; with a changing climatic regime in western North America, the surviving Taxodium-hardwood forest has been confined to southeastern North America. In Asia Taxodium is rare or absent in Pliocene floras; here Metasequoia has continued down to the present, though it is now confined to a limited area where environmental factors are favorable. This tree is reproducing on a limited scale, but its continued existence will be determined by the pressure put upon it by a people whose land, fuel and timber resources are wholly inadequate. A conservation committee has recently been organized in Nanking, and we may hope for its success in protecting some of the groves in which Metasequoia and its associates are living. Glyptostrobus, a close relative of Taxodium, has survived in the lowlands of South China. Sequoia, the living genus most similar to Metasequoia, is likewise a relict genus, restricted to the California and Oregon coast. Here the rainfall regime is wholly different from that in Central China, though the temperature, somewhat lower at this more northerly latitude, resembles that of western Szechuan in its equability (table 1). Our present knowledge of the Tertiary floras of Europe indicate that Heer was correct in his original reference of material from the Miocene of Switzerland to this genus, and that Sequoia rather than Metasequoia lived on this continent during the Tertiary period. Wide-spread submergence in western Europe at this time appears to have provided living conditions which favored Sequoia, although Metasequoia may be found in the fossil record of the Tertiary interior, farther to the east.

Having in mind the deciduous habit of Metasequoia and Taxodium, the deciduous hardwood associates of these genera, both in living forests and in the fossil record, and the modern environments in Central China and southeastern North America, we may suggest certain physical conditions

which may have been best suited to the origin and development of the Arcto-Tertiary Flora. A summer-wet climate appears to have been the primary requisite, with a total annual rainfall in excess of 40 inches. Moderate temperature seldom falling below freezing is also indicated, though a winter characterized by lower temperatures is indicated by the prevailing deciduous habit. The apparent absence of polar ice-caps during at least the early part of the Tertiary period, and postulated ocean circulation, makes it reasonable to accept the occurrence of Metasequoia and its associates as far north as Grinnell Land; possibly it was the darkness of the arctic night rather than low winter temperatures which brought about the annual shedding of the leaves of these trees, a habit which has been retained in Metasequoia even though winter temperature in its habitat in Central China seems not to make the deciduous habit a modern necessity. It is doubtful whether the present altitude of the Metasequoia occurrence, at about 4000 feet, has always been an essential requirement. More probable is the assumption that the high valleys occupied by these trees provide the only existing habitat which is suitable. Farther to the north at lower altitudes, extremes of temperature or precipitation characterize the climate of China, and although the fossil record shows that Metasequoia has lived there in the past, it can no longer do so. The limited area in eastern Szechuan and western Hupeh where the trees are known to have survived is surrounded by mountain ranges which protect them from the climatic extremes which characterize other inland environments. An equable climate rather than a high altitude appears to be the determining factor in the modern occurrence of Metasequoia.

We may summarize our current knowledge of the fossil occurrence and history of the genus Metasequoia as follows:

(1) Many of the fossil leaves and cones from the Cretaceous and Tertiary rocks of high northern latitudes, and from middle latitudes in North America and Asia, which have previously been referred to Sequoia and Taxodium, are now known to be Metasequoia on the basis of recent descriptions of fossil and living members of this genus in Asia.

(2) Vegetative and reproductive units in the fossil record differ in no essential respect from those of the living *M. glyptostroboides* of Central China. Like those of this tree, they are characterized by the decussate arrangement of their shoots, leaves and scales, and by the deciduous habit of leafy and cone-bearing shoots.

(3) Occurrence of Metasequoia in the Arcto-Tertiary Flora, at high latitudes in the Eocene epoch, at middle latitudes in the Oligocene and Miocene, provides evidence of the northern origin of this Flora, and of its southward migration during the Tertiary period.

(4) Survival of Metasequoia in Central China, in association with many of the hardwood genera recorded with it in the fossil records of the

northern hemisphere, provides a basis for reconstructing the climatic conditions under which the Arcto-Tertiary Flora had its origin and subsequent migration. A humid climate with summer rainfall, and with moderate temperatures not regularly falling below freezing, may be suggested as best suited to this deciduous forest during its past history.

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# INFLUENCE OF AMINO ACIDS ON GROWTH OF DATURA EMBRYOS IN CULTURE*

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Investigations of the growth of Datura embryos in culture have shown that as early as the preheart stage, the embryos can be excised from the seeds and grown in an artificial medium containing only the major inorganic salts and sucrose. Such cultured embryos sometimes showed premature differentiation of root or plumule and grew to the seedling stage in less time than is required for the normal cycle of capsule ripening and seed germination. However, initial growth of the excised embryos, from preheart to mature embryo, never equalled that of the corresponding period of development in the seed which covers from 10 to 14 days (Sanders[12, 13]). Addition of certain vitamins and organic acids did not markedly affect growth. However, addition of some organic complexes, notably coconut milk and malt extract, definitely increased the growth rate and favored normal development of the embryos (van Overbeek, Conklin and Blakeslee[16]; Blakeslee and Satina[1]).

Studies on the nitrogen metabolism of higher plants have shown that many plants grown in sand culture, especially if deprived of inorganic nitrogen, can absorb and utilize certain sources of organic nitrogen. Legumes have been found better able to use organic nitrogen than other plants, such as wheat and barley (Virtanen and Linkola[17]). Plants furnished with external supplies of different amino acids have been found to have distinct morphological variations (Virtanen and Linkola[17]; Steinberg[15]). Tomato and tobacco seedlings deprived of inorganic sulphur were found able to use DL-methionine as a source of sulphur (Miller[8]). Though many amino acids can be utilized, toxicity is frequent and may be due either to specific acids or to unfavorable concentrations. Under controlled optimum conditions for growth, ammonium nitrogen has been shown to be utilized more rapidly than nitrate, but under usual conditions for growth, nitrates have proved to be the best external source of nitrogen for higher plants (Nightingale[9, 10]).

With the development of tissue culture techniques, it has been possible to show that plant tissues, organs and embryos can assimilate inorganic nitrogen. On the whole, nitrates have again proved to be the best source of nitrogen although ammonium salts have been found available and, in some cases, a good source. For the most part, nitrogenous organic compounds have been toxic to plant tissue cultures, but there are exceptions. With tomato roots, White[19] found that glycine with thiamin satisfactorily

replaced yeast extract in the nutrient. For growth of bacteria-free crown-gall tissue of sunflower, Riker and Gutsche[11] found urea to be the only organic nitrogenous compound approaching the effectiveness of nitrate. Some of the amino acids (DL-alanine, L-arginine HCl, L and DL-aspartic acid and L-glutamic acid) did prove to be available for growth and were not toxic when present with nitrate. In the culture of orchid embryos, addition of amino acids to a medium containing nitrate failed to improve growth and frequently showed toxicity (Withner[20]; Spoerl[14]). Spoerl found that arginine and aspartic acid under certain conditions were as effective as ammonium nitrate, but used in addition to ammonium nitrate, they showed no stimulation of growth. He found that the effect of the amino acids depended upon concentration, light conditions, species of orchid and age of embryos. In studying the problem of rooting in asparagus stem tips in artificial culture, Galston[4] found that glutamine and arginine stimulated growth but were ineffective for root formation. Kent and Brink[6] observed that casein hydrolysate,‡ an amino acid complex, prolonged embryonic growth and inhibited germination of Hordeum embryos *in vitro*. Although the majority of amino acids supplied to plant cultures have proved to be growth inhibitors, alanine, arginine, aspartic acid, glutamic acid and glycine have proved beneficial to growth under certain conditions.

*Materials and Methods.*—Plants used in these investigations were *Datura inoxia* (S.I. 115) and *D. stramonium* (S.I. 1) (from the collection of Dr. A. F. Blakeslee) and were grown under greenhouse conditions. Self embryos were selected at the pre- or early heart stage (longitudinal axis, 0.1 to 0.25 mm.), dissected out of the seeds and grown in artificial liquid nutrients. Ten embryos per nutrient were used. Experiments ran for twelve days and results for individual media are expressed as growth values, final average length/original average length.

Basal media consisted of water, major inorganic salts, trace elements, vitamins and sucrose. The water used was double-distilled in Pyrex glass. The major salt formula was composed to obtain the following proportions of ions, 8 K : 1 Ca : 1 Mg and 4 $NO_3$ : 3 $PO_4$ : 3 $SO_4$. The following salts were used with "1" representing 1 millimolecular weight per liter of solution: 2 $KNO_3$, 1 $Ca(NO_3)_2$, 2 $KH_2PO_4$, 1 $NaH_2PO_4$, 1 $MgSO_4$ and 2 $K_2SO_4$. In some of the experiments, the inorganic nitrogen was increased so that the ratios were 8 K:1 Ca:1 Mg and 6 $NO_3$:3 $PO_4$:1 $SO_4$, and the formula became 4 $KNO_3$, 1 $Ca(NO_3)_2$, 3 $KH_2PO_4$, 1 $MgSO_4$ and 1 KCl. Increased nitrate, however, did not affect the growth values to any extent. The former formula gave growth values of 6.21 for *D. inoxia* and 5.5 for *D. stramonium;* the latter, 6.45 for *D. inoxia* and 4.88 for *D. stramonium*. Trace elements provided were in p. p. m.: B, 0.1; Mn, 0.1; Zn, 0.3; Cu, 0.1; Mo, 0.1; Fe, 0.5. Vitamin concentrations were in p. p. m.:

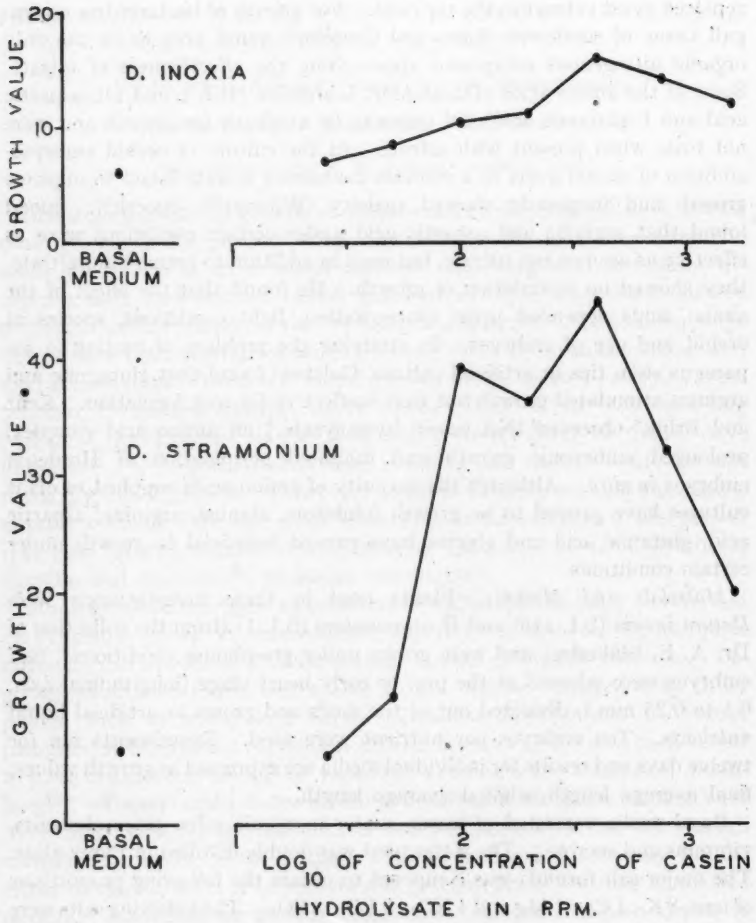


FIGURE 1

Response of preheart embryos of two species of Datura to casein hydrolysate with cysteine and tryptophane added to the basal medium.

thiamin HCl, 0.1; pyridoxine HCl, 0.8; niacin, 0.8. Sucrose was used at 2 per cent for *D. inoxia* and 4 per cent for *D. stramonium* (Sanders[13]). All nutrients were adjusted to between pH 5.7 and 6.0.

*Response of Embryos to Casein Hydrolysate with Cysteine and Tryptophane Added to the Basal Medium.*—Casein hydrolysate together with cysteine and tryptophane (each at 6.67 mg. per 100 mg. casein) was added to the

basal medium at 0, 25, 50, 100, 200, 400, 800 and 1600 p. p. m. Growth values for this series are represented in the graphs of figure 1. Growth of embryos of both species increased markedly especially in the range 100 to 800 p. p. m. Embryos of *D. stramonium* were much more stimulated than those of *D. inoxia*. At 400 p. p. m., the concentration which gave best growth in both species, growth values were 44.8 and 16.1, respectively.

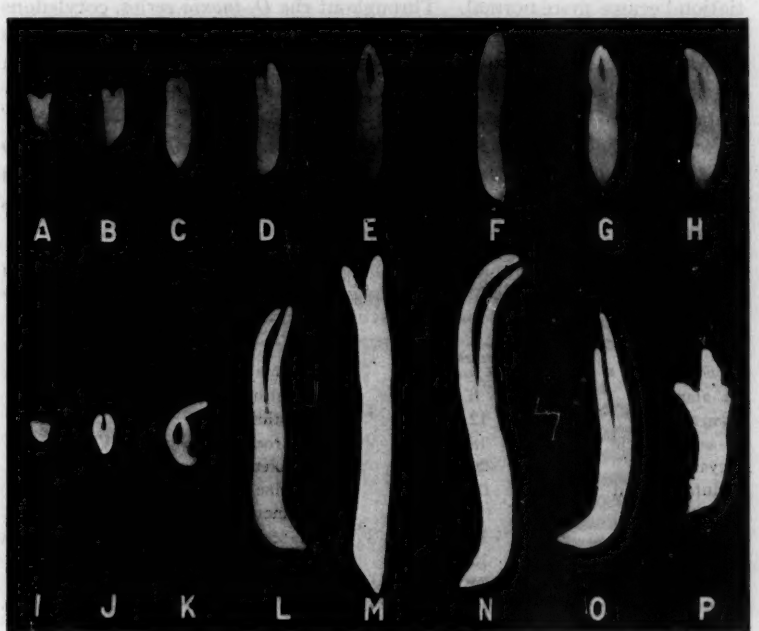


FIGURE 2

Photographs (× 5½) of Datura embryos grown for twelve days in a nutrient series to which casein hydrolysate with cysteine and tryptophane was added. *A–H*, embryos of *D. inoxia*. *I–P*, embryos of *D. stramonium*. *A* and *I*, in basal medium; *B* and *J*, in basal medium plus casein hydrolysate at 25 p. p. m.; *C* and *K*, at 50 p. p. m.; *D* and *L*, at 100 p. p. m.; *E* and *M*, at 200 p. p. m.; *F* and *N*, at 400 p. p. m.; *G* and *O*, at 800 p. p. m.; *H* and *P*, at 1600 p. p. m.

At 25 and 50 p. p. m., growth values were not much larger than for the basal medium, and at 800 and 1600 p. p. m., they decreased markedly in *D. stramonium* and somewhat in *D. inoxia*. Representative embryos from these series are shown in figure 2. The amino acid complex increased embryonic differentiation as well as the actual size of the embryos. After twelve days in basal medium, embryos were approximately 1 mm. in length. Embryos grown to this size in the seed have arrived at the late heart or

early torpedo stage and have well-defined cotyledons and no root primordia (Sanders[12]). In basal medium, *D. inoxia* embryos showed retarded cotyledonary development and frequently premature root primordia, and *D. stramonium* embryos showed little differentiation although cotyledon primordia were sometimes discernible (figure 2, *A* and *I*). With addition of casein hydrolysate, even at the lower concentrations, embryonic differentiation became more normal. Throughout the *D. inoxia* series, cotyledons were shorter in relation to the hypocotyl than are cotyledons of embryos grown in the seed. Abnormalities and root primordia appeared when casein hydrolysate was present, but less frequently and on much larger embryos than in basal medium.

*Response of Embryos to Mixtures of Amino Acids and to Ten Single Amino Acids Added to Basal Medium.*—In an attempt to identify the growth factors in casein hydrolysate, embryos were grown in a series of nutrients

TABLE 1

COMPOSITION OF AMINO ACID MIXTURES ADDED TO BASAL MEDIUM USED FOR GROWTH OF *Datura* EMBRYOS. (ACIDS MARKED WITH AN ASTERISK WERE THE COMPONENTS OF "MODIFIED MIXTURES" A AND B)

| MIXTURE A | | MIXTURE B | |
|---|---|---|---|
| Amino Acid | Concentration, p. p. m. | Amino Acid | Concentration p. p. m. |
| DL-alanine | 6.0 | *L-cystine | 1.5 |
| *L-arginine | 12.0 | *L-histidine | 9.0 |
| *L-aspartic acid | 18.0 | L-hydroxyproline | 1.5 |
| L-cysteine | 20.0 | DL-isoleucine | 15.0 |
| L-glutamic acid | 60.0 | L-leucine | 15.0 |
| Glycine | 1.5 | *DL-methionine | 9.0 |
| *L-lysine | 18.0 | *DL-serine | 18.0 |
| DL-phenylalanine | 12.0 | DL-threonine | 12.0 |
| *L-proline | 24.0 | L-trytophane | 20.0 |
| L-tyrosine | 18.0 | *DL-valine | 24.0 |

containing mixtures of amino acids or individual amino acids. Three controls were run for the series, basal medium, basal medium with $NH_4Cl$ at 108 p. p. m. (an amount roughly equivalent to the amino nitrogen in casein hydrolysate at 300 p. p. m.), and basal medium plus casein hydrolysate at 300 p. p. m. with L-cysteine and L-tryptophane. Concentrations of individual amino acids (with the exception of cysteine and tryptophane which were used at the previous concentrations given above), were based on the amino acid analysis of casein hydrolysate (Harrow[5]). Amounts approximately equivalent to the content of the acids in hydrolyzed casein at 300 p. p. m. were determined. The amino acids were divided into two groups of ten each; mixture A included those acids which have been found beneficial for plant growth under certain conditions (Burkholder, Castle and Smith[3]; Riker and Gutsche[11]; Spoerl[14]; White[19]); mixture B, the remaining ten acids which have shown no effect or have been

inhibitory to growth. The composition of mixtures A and B are given in table 1. Acids marked with an asterisk are those which were included in modified mixtures A and B. Amino acids of mixture A were also added singly to the basal medium at the same concentrations as in the mixture. Data from this series of nutrients are represented by the graphs in figure 3. In both species, addition of ammonium chloride increased growth somewhat suggesting an ammonium salt might be beneficial in the basal medium, but it did not approach the stimulatory effect of casein hydrolysate. Addition of mixtures A and B together in the same medium closely reproduced the results obtained with casein hydrolysate, strongly suggesting that the growth stimulus is due to the amino acids present in hydrolysed

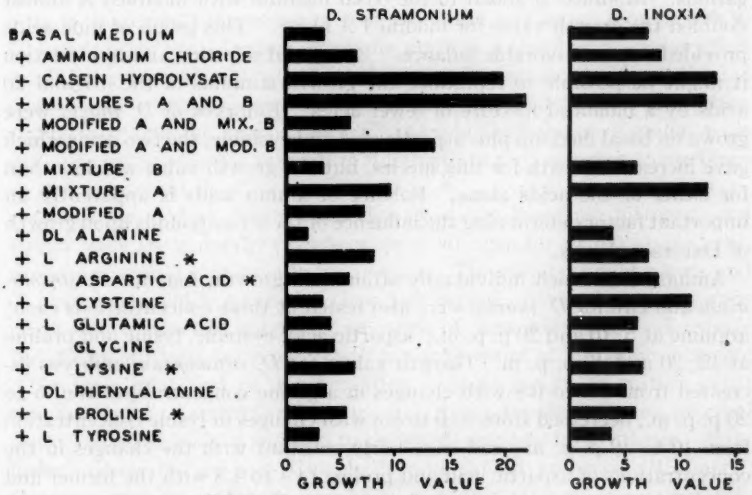


FIGURE 3

Response of preheart embryos of two species of Datura to ammonium chloride and various organic nitrogen sources added to the basal medium.

casein. Addition of B alone gave no increase in growth. Addition of A alone gave a growth value for *D. inoxia* embryos equal to that for A and B, suggesting that the entire stimulatory effect of casein hydrolysate might be contributed by the amino acids in A. The growth value for *D. stramonium* embryos on basal medium with A alone was less than half that with A and B suggesting that an interaction of the acids in the two mixtures is necessary to produce the growth stimulus obtained with casein hydrolysate. Addition of single amino acids also gave different results for the two species. With *D. inoxia* embryos, cysteine alone gave a definite increase in growth; aspartic acid showed a slight increase; alanine, phenylalanine, tyrosine

and perhaps glycine showed inhibition. With *D. stramonium* embryos, arginine, aspartic acid, lysine and proline increased growth; alanine and perhaps tyrosine showed inhibition. Modified mixture A was made up of the four acids stimulatory to *D. stramonium* embryos when added separately. Comparison of the growth value for modified A with that for A indicates that at least some of the six omitted acids were functional in promoting growth either individually or by interaction with other acids. Mixture B added with modified A decreased rather than increased embryo growth. Omission of six acids from A has apparently upset the amino acid balance which favored growth. Modified mixture B was made up by omitting five of the acids in B which have shown inhibition in other investigations. Modified B added to the basal medium with modified A almost doubled the growth value for modified A alone. This group of nine acids provided a more favorable balance. By careful selection and combination it might be possible to reproduce the growth stimulus of the original 20 acids by a balanced mixture of fewer acids. Embryos of *D. inoxia* were grown on basal medium plus aspartic acid and cysteine, the two acids which gave increased growth for this species, but the growth value was less than for either of the acids alone. Balance of amino acids is apparently an important factor determining the influence of these compounds upon growth of Datura embryos.

Amino acids which individually stimulated growth, four for *D. stramonium* and two for *D. inoxia*, were also tested at three concentrations each; arginine at 5, 10 and 20 p. p. m.; aspartic acid, cysteine, lysine and proline at 10, 20 and 40 p. p. m. Growth values for *D. stramonium* embryos increased from 3.3 to 6.8 with changes in arginine concentration from 5 to 20 p. p. m., decreased from 10.0 to 6.5 with changes in lysine concentration from 10 to 40 p. p. m., and were fairly constant with the changes in the concentrations of aspartic acid and proline (4.4 to 4.8 with the former and 6.1 to 5.6 for the latter). Growth values for *D. inoxia* embryos were 6.9, 8.9 and 6.9 with aspartic acid at 10, 20 and 40 p. p. m., and increased from 5.0 to 13.7 with cysteine from 10 to 40 p. p. m. Interaction of the acids casts some doubt on the possibility of determining optimum concentrations for growth by addition of varying amounts of single acids. This procedure does provide some evidence concerning the effect of different concentrations of the individual amino acids on growth and differentiation.

It has been mentioned that addition of casein hydrolysate to the basal medium favored normal differentiation of the embryos in culture. Mixtures A and B together were equally beneficial to normal differentiation. The other mixtures and the single amino acids were less effective for normal differentiation, but certain characteristics appeared. Although growth values for *D. inoxia* embryos in basal medium with A plus B and with A alone were very similar, the patterns of growth differed. With A plus B,

cotyledons of the embryos were short in proportion to the hypocotyls as with casein hydrolysate (figure 2, *B–H*). With A alone, hypocotyls were short in proportion to cotyledons. Addition of mixture A resulted in the same type of growth in *D. stramonium* embryos whereas with A plus B, cotyledon-hypocotyl proportions were normal as in figure 2, *L–O*. Modified A had the same effect as A, but modified A plus modified B produced embryos with proportionally short cotyledons. Differentiation as well as growth is influenced by the interaction of the amino acids present. Some single amino acids had recognizable effects on type of growth. Alanine interfered with cotyledon and hypocotyl differentiation in *D. inoxia* embryos but allowed formation of premature root primordia and even slight growth in the region of the plumule. Cysteine at 20 and 40 p. p. m. increased the number of root primordia and actual root growth in *D. inoxia* embryos. Lysine, especially at 10 p. p. m., had a similar effect on *D. stramonium* embryos. It also contributed to the formation of translucent, watery tissue instead of the firm, white tissue of healthy embryos beyond the heart stage. Changes in the balance of amino acids can apparently enhance premature differentiation of embryos to seedlings as well as prevent it.

*Discussion.*—Most plants naturally assimilate inorganic nitrogen, and amino acids are generally poor sources of nitrogen for plants in sand cultures and for many plant cultures *in vitro*. The metabolism of plants and of plant parts which synthesize their own organic nitrogenous compounds is apparently upset by supplying an excess of these substances. To study the effects of amino acids on growth in the presence of inorganic nitrogen, other than inhibition or abnormalities, it is necessary to culture plant tissues, organs or embryos which are able to use to advantage an external source of amino acids in addition to their own capacity for synthesis. Sunflower tissue (Riker and Gutsche[11]) and orchid embryos (Withner[20]; Spoerl[14]) with an adequate supply of inorganic nitrogen are apparently able to synthesize their necessary amino acids as rapidly as they can use them. Asparagus stem tips (Galston[4]), tomato root tips (White[19]), immature Hordeum embryos (Kent and Brink[6]) and immature Datura embryos can apparently use amino acids faster than some of these compounds can be synthesized in such isolated structures. It may well be that *in vivo* amino acids are supplied to such structures by other parts of the plant. Without an external source of amino acids, young Datura embryos grown in culture require a much longer time to mature than in the seed or with a mixture of amino acids in the nutrient.

The behavior of mixtures indicates that the influence of these acids on growth results from interaction rather than accumulation of the individual effects of single acids. Evidence of such interaction has been found with numerous microorganisms. For example, Bonner[2] demonstrated in a

single gene mutant of Neurospora that a block in the conversion of keto isoleucine to isoleucine resulted in an accumulation of the former which inhibited the conversion of keto valine to valine, thus causing a deficiency of two amino acids. Inhibition of growth in *Lactobacillus arabinosus* by glycine can be counteracted by the addition of D-alanine (Kobayashi, Fling and Fox[7]). Inhibition of growth in *Streptococcus bovis* by isoleucine, leucine, threonine or norleucine can be counteracted by valine, glutamic acid, methionine or cystine. Inhibition caused by equal amounts of phenylalanine and tyrosine, which singly had no effect, can be counteracted by tryptophane, glutamic acid or cystine. It is suggested that inhibiting amino acids may block the synthesis of another acid and that the counteracting acid may be the acid blocked or an acid from which it can be formed (Washburn and Niven[18]).

Single additions of amino acids have been found to have characteristic effects on the morphology of plants. This phenomenon suggests that the balance of amino acids probably changes during development and that such changes may influence growth and differentiation. Steinberg[15] suggests changes in the balance of amino acids as the basis for the production of morphological symptoms of certain plant diseases. Some of the problems of differentiation, relative growth and dormancy, would appear to be closely related to amino acid metabolism.

The effect on embryo growth of casein hydrolysate and of the mixture of amino acids approximating the composition of casein hydrolysate is similar to the beneficial effects of other organic complexes, namely coconut milk and malt, which have been found to stimulate growth of Datura embryos, favor embryonic differentiation and retard premature germination (van Overbeek, Conklin and Blakeslee[16]; Blakeslee and Satina[1]). The varied activities of such natural complexes may well be due to their component amino acids.

Differences in the effects of amino acid mixtures and of single acids on embryos of two separate species suggest that the amino acid content of female parent tissues and of endosperm may be important in interspecific crosses, especially crosses in which embryos are first formed but later abort. An attempt to correlate similarity of amino acid requirements of self embryos of the parent species and of the hybrid embryos with species crossability might prove more successful than earlier attempts to correlate species crossability with similarity of sucrose and inorganic salt requirements of the embryos (Sanders[13]).

*Summary.*—Early heart embryos of *Datura inoxia* and *D. stramonium* (0.1 to 0.25 mm. in length) were dissected out of the seeds and grown in artificial culture in basal medium alone and also with additions of casein hydrolysate, mixtures of amino acids, and single amino acids.

Casein hydrolysate with cysteine and tryptophane markedly improved growth and embryonic differentiation in the range 100 to 800 p. p. m.

A mixture of 20 amino acids approximating their occurrence in casein hydrolysate at 300 p. p. m. resulted in growth equal to that with casein hydrolysate indicating that the amino acids are responsible for the growth stimulus provided by hydrolyzed casein.

Differences in growth values for lesser mixtures and for individual amino acids, which were for the most part well below those for the mixture of 20 acids, provide evidence that the growth stimulus of the combination of 20 acids results from physiological interactions rather than from the summation of effects of individual acids.

Characteristic patterns of differentiation appeared with some mixtures and with some individual acids.

The species differed in their responses both to mixtures and to single amino acids.

Balance of amino acids appears to be a controlling influence on both growth and differentiation of plant embryos.

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‡ From General Biochemicals, Inc., Chagrin Falls, Ohio.

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## *ON THE STABILITY AND INSTABILITY OF SHOCK WAVES*

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The following communication contains a criterion for the stability and instability of plane stationary shock waves (compression shocks) attached to the vertex $V$ of a pointed obstacle $O$ in a uniform field of flow.[1] Behind the shock line the flow may be rotational and non-isentropic; viscosity and thermal conductivity are assumed zero in this theory. It will be assumed that the shock line and boundary of the obstacle are analytic

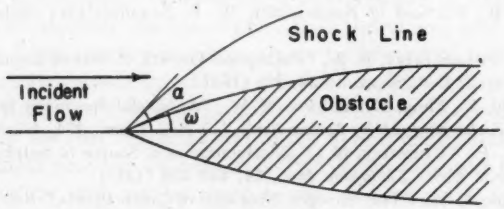


FIGURE 1

curves and that the functions $u_\alpha$ (velocity components), $p$ (pressure) and $\rho$ (density) defining the flow are analytic in the region behind, and also along the shock line.

Angles $\alpha$ and $\omega$ give the inclination of the shock line and obstacle at $V$ relative to the direction of the incident flow. Attention may be confined in the following discussion to the "upper half" of the shock line as indicated in the figure. The range of permissible values of $\alpha$ is given by $\alpha_0(M) < \alpha \leqq 90°$ where $M(>1)$ is the mach number of the incident flow and $\alpha_0(M)$ is the value of $\sin^{-1} 1/M$ in the first quadrant. Denote by $s$ the arc length along the shock line, measured toward $V$, and by $\eta$ the arc length along the obstacle measured in the direction of flow. Let $K$ and $\kappa$ be the curvatures of the contour of the obstacle and shock line, respectively, and put $K_{(n)} = d^nK/d\eta^n$, $\kappa_{(n)} = d^n\kappa/ds^n$ for $n = 0, 1, 2, 3, \ldots$ so that $K_{(0)} = K$ and $\kappa_{(0)} = \kappa$. It can be shown that there exist relations of the form[2]

$$K_{(n)} = G_n(M, \alpha)\kappa_{(n)} + H_n; \; n = 0, 1, 2, 3, \ldots, \qquad (1)$$

at point $V$, where the $H_n$ are polynomials in $\kappa$ and its derivatives with respect to $s$ of order less than $n$. The quantities $G_n(M, \alpha)$ and the coefficients of the polynomials $H_n$ are rational functions of $M^2$ with coefficients depending on the inclination $\alpha$ of the shock line. In particular for $n = 0$ the equation (1) becomes $K = G_0(M, \alpha)\kappa$. Actually the relations (1) hold at any point of the shock line when $\alpha$ is the inclination of the shock line and $K$ the curvature of the stream line behind the shock line at this point but we shall be concerned primarily with these relations at the vertex $V$ of the obstacle. *For a given value of $M > 1$, a value of $\alpha$ for which any one of the functions $G_n(M, \alpha) = 0$ will be said to be singular relative to $M$.*

The above relations (1) appear as *consistency conditions* for the shock wave solution of the hydrodynamical problem. If a shock wave solution exists for a singular value of $\alpha$, corresponding to $G_i(M, \alpha) = 0$, then any variation in the value of $K_{(i)}$ will result in an inconsistency among the relations (1). Hence when $\alpha$ is singular there will exist in general no shock wave solution under the assumptions of continuity and differentiability involved in the derivation of the relation (1) for which the coefficient $G_i(M, \alpha)$ vanishes.

Denoting by $m$ the mach number for the flow behind the shock line at $V$ there exists a number $\beta = \beta(M)$ such that $\alpha_0(M) < \beta(M) < 90°$ and such that $m = 1$ for $\alpha = \beta(M)$. Furthermore $m > 1$ for $\alpha_0(M) < \alpha < \beta(M)$ and $m < 1$ for $\beta(M) < \alpha \leqq 90°$. *It can be shown that the zeros of the functions $G_n(M, \alpha)$,* i.e., *the singular shock angles $\alpha$ relative to any mach number $M > 1$, lie entirely within the interval $\beta(M) < \alpha \leqq 90°$ and are in fact dense in this interval.*

The figure shows the nature of the graph of the angle $\omega$ for a given value of $M > 1$ and the location of the singular shock interval. Point $A$ corresponds to the value $\alpha = \alpha_0(M)$ and $B$ to the value $\alpha = \beta(M)$ at which $m = 1$. In the open interval $AB$ no singular shock angles $\alpha$ occur, nor is there a singular shock angle at the end-point $B$. The singular shock

angles are dense in the open interval $BD$ and a singular angle occurs at $D$. The $\omega$ curve reaches its maximum height at a point $E$, corresponding to a value of $\alpha$, represented by the point $C$, in the singular shock interval.[3]

The following criterion for the stability and instability of attached shock waves is based on the above stated results. Let $\bar{O}$ be an obstacle, analogous to the obstacle $O$, having inclination $\bar{\omega}$, curvature $\bar{K}$ and derivatives of curvature $\bar{K}_{(n)}$ at its vertex $V$. Then $\bar{O}$ will be said to be in a $\delta$ neighborhood of $O$ provided that $|\omega - \bar{\omega}| < \delta$ and $|K_{(n)} - \bar{K}_{(n)}| < \delta$ for $n = 0, 1, 2, 3, \ldots$. Also the shock wave $\overline{W}$ for the flow past the obstacle $\bar{O}$ will be said to be in the $\epsilon$ neighborhood of the shock wave $W$ for the flow past $O$ when $|\alpha - \bar{\alpha}| < \epsilon$. On the basis of these definitions we now lay down the following definition of *local stability* or stability at the vertex $V$; in this statement we are concerned only with the contour of the obstacle, the

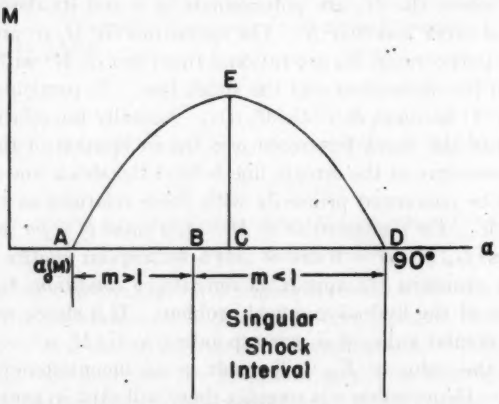


FIGURE 2

shock wave and the hydrodynamical solution in the immediate neighborhood of $V$. *The shock wave attached to the vertex of the obstacle $O$, or the associated hydrodynamical solution, will be said to be locally stable or stable at $V$, relative to the incident flow, provided that for any $\epsilon > 0$ there can be found a $\delta > 0$ such that solutions with shock waves $\overline{W}$ in the $\epsilon$ neighborhood of $W$ exist for all obstacles $\bar{O}$ in the $\delta$ neighborhood of $O$. Otherwise the shock wave attached to the vertex of the obstacle $O$ (or the associated solution) will be said to be locally unstable or unstable at $V$.*

Suppose that the shock angle $\alpha$ lies in the interval $\beta(M) \leqq \alpha \leqq 90°$ for the flow past the obstacle $O$. Then if $\delta$ and $\epsilon$ are any two positive numbers we can find an inclination angle $\bar{\omega}$ and corresponding singular shock angle $\bar{\alpha}$ such that $|\omega - \bar{\omega}| < \delta$ and $|\alpha - \bar{\alpha}| < \epsilon$; this follows immediately from the fact that the singular values $\alpha$ are dense in the above interval and $\omega$ is a continuous function of $\alpha$. Now consider the obstacle $\bar{O}$, with

vertex at $V$, having inclination $\bar{\omega}$ and for which $\bar{K}_{(n)} = K_{(n)}$ ($n = 0, 1, 2, 3\ldots$) at $V$. In general the set of relations (1), in which $\alpha$ is replaced by the above singular value $\bar{\alpha}$, will be inconsistent for the obstacle $\bar{O}$. If, however, these relations are consistent a slight variation in the value of $\bar{K}_{(j)}$, assuming $G_j(M, \bar{\alpha}) = 0$, while the values of all other $\bar{K}_{(n)}$ remain unchanged, will result in a new obstacle $\bar{O}$ for which the relations (1) are inconsistent. Hence, there can be found an obstacle $\bar{O}$ in any $\delta$ neighborhood of $O$ for which no attached shock wave solution exists. *In other words a shock line for which the inclination $\alpha$ lies in the interval $\beta(M) \leqq \alpha \leqq 90°$ is unstable at $V$ (also unstable in the large from the following discussion).*

In the special case of flow past an infinite wedge two shock wave solutions are possible. Here the shock lines are straight lines and the flow behind the shock is uniform. Contrary to the general situation solutions now exist for all singular values of the shock angle $\alpha$. However, those solutions for which the shock angle $\alpha$ lies within the interval $\beta(M) \leqq \alpha \leqq 90°$, or, in other words, for which $m \leqq 1$ (where $m$ is the mach number for the flow behind the shock) will be unstable.

If the shock angle $\alpha$ lies in the interval $\alpha_0(M) < \alpha < \beta(M)$ so also will $\bar{\alpha}$ for $|\bar{\alpha} - \alpha| < \epsilon$ and $\epsilon$ sufficiently small. Corresponding to this value of $\epsilon$ we can find a $\delta$ such that for all inclination angles $\bar{\omega}$, for which $|\bar{\omega} - \omega| < \delta$, the associated shock angles $\bar{\alpha}$ will be in the interval $|\bar{\alpha} - \alpha| < \epsilon$. Hence for any obstacle $\bar{O}$, in the $\delta$ neighborhood of $O$, the associated shock angle $\bar{\alpha}$ will lie in the non-singular interval $|\bar{\alpha} - \alpha| < \epsilon$ and hence the formal power series determination, in the neighborhood of $V$, of the shock line and of the functions $u_{\bar{\alpha}}$, $p$ and $\rho$ behind the shock line will be possible. The proof of local stability requires only the convergence of these series for obstacles $\bar{O}$, with analytic contours, in the arbitrarily small $\delta$ neighborhood of the obstacle $O$ for which the corresponding series are assumed to converge. Presumably these series will converge under this condition with the result that the shock line will be stable at $V$ when the shock angle $\alpha$ lies within the interval $\alpha_0(M) < \alpha < \beta(M)$. It is not our intention, at present, to carry out the details of this convergence proof.

The concept of local stability has been introduced as a step toward the solution of the more proper, but also more complicated, problem of *stability in the large.* Conditions for stability in the large are to be phrased in an analogous manner except that we must here be concerned with the entire extent of the shock line, and solution of the hydrodynamical problem behind the shock line, including the conditions at infinity. In this connection it may be advisable to confine our attention in certain circumstances to a special class of obstacles, e.g., obstacles with analytic contours reaching to infinity and concave downward at all points of the portion of the contour under consideration, and so arrive at a concept of

stability relative to this special class. Moreover the problem can be extended, within the framework of our general method, by considering obstacles whose contours are composed of a finite number of analytic arcs with shock waves originating at their points of intersection.

*It is evident that instability at V, or local instability, is sufficient to insure instability in the large.* Hence, the above result on instability gives the complete answer to the problem of determining the conditions for instability of shock lines attached to the vertex $V$ of an obstacle whose contour is an analytic curve. Since at most two shock angles $\alpha$ at $V$ are mathematically possible the shock line which actually occurs and which corresponds to the shock line experimentally observed must therefore be the one whose inclination $\alpha$ lies in the interval $\alpha_0(M) < \alpha < \beta(M)$. This may be accepted as sufficient evidence for the stability (local or in the large) of shock lines with inclination $\alpha$ in the interval $\alpha_0(M) < \alpha < \beta(M)$ by those not interested in an existence-theoretic treatment of the problem.

[1] Prepared under Navy Contract N6onr-180, Task Order V, with Indiana University.

[2] The derivation of these relations and other results mentioned in this note are contained in several papers which we expect to publish later in the *Journal of Mathematics and Physics* under the following titles: "Calculation of the Curvatures of Attached Shock Waves"; "The Consistency Relations for Shock Waves"; and "The Distribution of Singular Shock Directions."

[3] This conclusion is reached by an observation of the graphs of the functions $\omega$ and $-G_0(M, \alpha)$ shown in the paper "Calculation of the Curvatures of Attached Shock Waves."

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## *PROGRESS IN THE STATISTICAL THEORY OF TURBULENCE**

BY THEODORE VON KÁRMÁN

CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA

Communicated August 2, 1948

The fundamental notion of statistical mean values in fluid mechanics was first introduced by Reynolds. His most important contributions were the definition of the mean values for the so-called Reynolds' stresses and the recognition of the analogy between the transfer of momentum, heat and matter in the turbulent motion.

In the decades following Reynolds' discoveries, the turbulence theory was directed toward finding semi-empirical laws for the mean motion by methods loaned from the kinetic theory of gases. Prandtl's ideas on momentum transfer and Taylor's suggestions concerning vorticity transfer belonged to the most important contributions of this period. I believe that my formulation of the problem by the application of the similarity principle has the merit to be more general and independent of the methods

of the kinetic theory of gases. This theory led to the discovery of the logarithmic law of velocity distribution in shear motion for the case of homologous turbulence.

The next important step was the definition of isotropic turbulence by Taylor and the following period in the development of the theory of turbulence was devoted to the analysis of the quantities which are accessible to measurement in a wind tunnel stream. These quantities are the correlation functions and the spectral function. The general mathematical analysis of the correlations was executed by L. Howarth and myself. One has to consider five scalar functions $f(r)$, $g(r)$, $h(r)$, $k(r)$, $l(r)$. These functions determine all double and triple correlations between arbitrary velocity components observed at two points because of the tensorial character of the correlations. The two scalar functions for the double correlations are defined as follows:

$$f(r) = \frac{\overline{u_1(x_1, x_2, x_3)u_1(x_1 + r, x_2, x_3)}}{\overline{u_1^2}}, \qquad (1)$$

$$g(r) = \frac{\overline{u_1(x_1, x_2, x_3)u_1(x_1, x_2 + r, x_3)}}{\overline{u_1^2}}.$$

Because of the continuity equation for incompressible fluids $g = f + \frac{r}{2}\frac{df}{dr}$.

For the same reason the triple correlations $h$, $k$ and $l$ can be expressed by one of them, e.g., by

$$h(r) = \frac{\overline{[u_1(x_1, x_2, x_3)]^2 u_1(x_1 + r, x_2, x_3)}}{[\overline{u_1^2}]^{3/2}}. \qquad (1a)$$

In addition we also deduced a differential equation from the Stokes-Navier Equation which gives the relation between the time derivative of the function $f$ and the triple correlation function $h$.

$$\frac{\partial}{\partial t}(f\overline{u^2}) + 2[\overline{u^2}]^{3/2}\left(\frac{\partial h}{\partial r} + \frac{4}{r}h\right) = 2\nu\overline{u^2}\left(\frac{\partial^2 f}{\partial r^2} + \frac{4}{r}\frac{\partial f}{\partial r}\right). \qquad (2)$$

We discussed this equation in two special cases:

(*a*) Small Reynolds number—in this case the triple correlations can be neglected and one obtains a self-preserving form for the double correlation function as a function of $r/\lambda$, where $\lambda$ is defined by the relation

$$\frac{d\overline{u^2}}{dt} = -10\nu\frac{\overline{u^2}}{\lambda^2}. \qquad (3)$$

(*b*) Large Reynolds number—in this case the terms containing the vis-

cosity can be neglected for not too small values of $r$ and the functions $f$ and $h$ are assumed to be functions of the variable $r/L$; $L$ is a length characterizing the scale of turbulence. The hypothesis of self-preserving correlation function leads to the following special results. One can consider three simple cases:

1. $L$ = constant; then we have $\overline{u^2} \sim t^{-2}$ (Taylor).
2. Loitziansky has shown that if the integral $\overline{u^2}\int_0^\infty r^4 f(r)dr$ exists, it must be independent of time, consequently $\overline{u^2}L^5$ = constant. Then $\overline{u^2} \sim t^{-10/7}$, $L \sim t^{2/7}$.
3. If the self-preserving character is extended to all values of $r$, i.e., also near $r = 0$, one has $\overline{u^2} \sim t^{-1}$, $L \sim t^{1/2}$ (Dryden).

On the other hand, Taylor introduced a spectral function for the energy passing through a fixed cross-section of a turbulent stream as the Fourier transform $\mathfrak{F}_0(n)$ of the correlation function $f(r)$. The relation between $\mathfrak{F}$ and $f$ is given by the following equations:

$$f(r) = \int_0^\infty \mathfrak{F}_0(n) \cos \frac{2\pi n r}{U} dn,$$
$$\mathfrak{F}_0(n) = \frac{4\overline{u^2}}{U} \int_0^\infty f(r) \cos \frac{2\pi n r}{U} dr. \tag{4}$$

In these equations $n$ is the frequency of the fluctuation of the uniform velocity $U$ as function of time. Relative to the stream, $\mathfrak{F}_0(n)$ can be replaced by $\mathfrak{F}_1(\kappa_1)$, where $\kappa_1 = \frac{2\pi n}{U}$, i.e., the wave number of the fluctuation, measured in the $x_1$ direction.

It is seen that in this period of the development of the turbulence theory the analytical and experimental means for the study of isotropic turbulence were clearly defined but (with the exception of the case of very small Reynolds numbers) no serious attempt was made to find the laws for the shapes of either the correlation or the spectral functions. I believe this is the principal aim of the period in which we find ourselves at present. Promising beginnings were made by Kolmogoroff, Onsager, Weizsäcker and Heisenberg. I do not want to follow the special arguments of these authors. I want rather to define the problem clearly and point out the relations between assumptions and results.

*A*—I will assume that the three components of the velocity in a homogeneous isotropic turbulent field, at any instant, can be developed in the manner of Fourier's integrals

$$u_i = \int_{-\infty}^{\infty} \int Z_i(\kappa_1, \kappa_2, \kappa_3, t) e^{i(\kappa_1 x_1 + \kappa_2 x_2 + \kappa_3 x_3)} d\kappa_1 d\kappa_2 d\kappa_3. \tag{5}$$

*B*—The intensity of the turbulent field be characterized by the quad-

ratic mean value $\overline{u_i^2}$ level of the turbulence). Also there exists a function $\mathfrak{F}(\kappa)$ such that $[\overline{u_i^2}]_0^\kappa = \int^\kappa \mathfrak{F}(\kappa')d\kappa'$, where the symbol $[\overline{u_i^2}]_0^\kappa$ means a partial mean value of the square of the velocity, the averaging process being restricted for such harmonic components whose wave numbers $\kappa_1$, $\kappa_2$, $\kappa_3$ satisfy the relation

$$\kappa_1^2 + \kappa_2^2 + \kappa_3^2 \leqq \kappa^2. \tag{6}$$

If such a function exists it is connected with the spectral function of Taylor $\mathfrak{F}_1(\kappa_1)$ by the relation

$$\mathfrak{F}_1(\kappa_1) = \frac{1}{4}\int_{\kappa_1}^{\infty} \frac{1}{\kappa^3}\mathfrak{F}(\kappa)(\kappa^2 - \kappa_1^2)d\kappa. \tag{7}$$

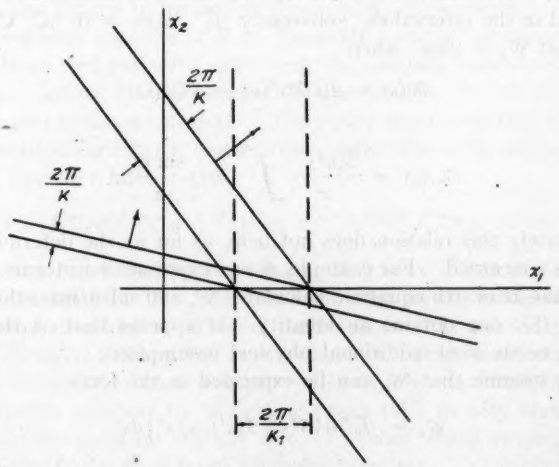


FIGURE 1

Contribution of oblique waves to plane waves in direction of $x_1$.

This relation was found by Heisenberg. It expresses the geometrical fact that all oblique waves, figure 1, whose wave-length $\frac{2\pi}{\kappa} < \frac{2\pi}{\kappa_1}$, necessarily contribute in the one-dimensional analysis to the waves with wave length $2\pi/\kappa_1$.

*C*—It is evident that there must be an equation for the time derivative of $\mathfrak{F}(\kappa)$ which corresponds to the equation for the time derivative of $f(r)$ which has been found by Howarth and myself. The physical meaning of this equation is evident. Let us start from the energy equation for a fluid element:

$$\frac{1}{2}\frac{\partial \overline{u_i^2}}{\partial t} + \overline{(u_i u_j + \delta_{ij} p/\rho)\frac{\partial u_i}{\partial x_j}} = \nu \overline{\frac{\partial^2 u_i}{\partial x_j^2} u_j}.$$

The right side represents the energy dissipation by viscous forces. The second term on the left side is the work of the Reynolds stresses; it represents a transfer of energy without actual dissipation. Our problem is to find $\partial \mathfrak{F}/\partial t$ by Fourier analysis and averaging process. One finds the contribution of the viscous forces to be equal to $-2\nu \mathfrak{F}(\kappa)\kappa^2$. Hence we write formally

$$\frac{\partial \mathfrak{F}}{\partial t} + \mathfrak{W}_\kappa = -2\nu\kappa^2 \mathfrak{F}(\kappa). \tag{8}$$

Here $\mathfrak{W}_\kappa d\kappa$ is the balance for the energy contained in harmonic components comprised in the interval $d\kappa$; obviously $\int_0^\infty \mathfrak{W}_\kappa d\kappa = 0$. C. C. Lin has shown that $\mathfrak{W}_\kappa = 2\mathfrak{K}\kappa^2$, where

$$\mathfrak{K}(\kappa) = 2(\kappa^2 \mathfrak{K}_1''(\kappa) - \kappa \mathfrak{K}_1'(\kappa))$$

and

$$\mathfrak{K}_1(\kappa) = \frac{2(\overline{u^2})^{3/2}}{\pi} \int^\infty h(r) \frac{\sin \kappa r}{\kappa} dr.$$

Unfortunately this relation does not help, as far as the determination of $f$ and $h$ is concerned. For example, if one expresses $h$ in terms of $f$ from the Kármán-Howarth equation, calculates $\mathfrak{W}_\kappa$ and substitutes the result in equation (8), one obtains an identity. It appears that at the present time one needs some additional physical assumption.

*D*—We assume that $\mathfrak{W}_\kappa$ can be expressed in the form:

$$\mathfrak{W}_\kappa = \int_0^\infty \Theta\{\mathfrak{F}(\kappa), \mathfrak{F}(\kappa'), \kappa, \kappa'\} d\kappa'. \tag{9}$$

The physical meaning of this assumption is the existence of a transition function for energy between the intervals $d\kappa$ and $d\kappa'$ which depends only on the energy density and the wave numbers of the two intervals. It follows from this definition that by interchanging $\kappa$ and $\kappa'$, one has

$$\Theta\{\mathfrak{F}(\kappa), \mathfrak{F}(\kappa'), \kappa, \kappa'\} = -\Theta\{\mathfrak{F}(\kappa'), \mathfrak{F}(\kappa), \kappa', \kappa\}. \tag{10}$$

It must be noted that our assumption probably cannot be exact. It is very probable that the values of $\mathfrak{F}$ for the difference and the sum of $\kappa$ and $\kappa'$ also enter in the transition function. I believe that the assumption gives a fair approximation when $\kappa$ and $\kappa'$ are very different, but it is certainly untrue if $\kappa$ and $\kappa'$ are nearly equal.

*E*—We furthermore specify the function $\Theta$ in the following way:

$$\Theta = -C\mathfrak{F}(\kappa)^\alpha \mathfrak{F}(\kappa')^{\alpha'} \kappa^\beta \kappa'^{\beta'}; \qquad C = \text{const.} \tag{11}$$

It follows from dimensional reasoning that

$$\alpha + \alpha' = 3/2, \qquad \beta + \beta' = 1/2.$$

As a result of the sequence of assumptions given above we obtain the equation:

$$\frac{\partial \mathfrak{F}}{\partial t} = C\left[ \mathfrak{F}^{\alpha}\kappa^{\beta} \int_0^{\kappa} \mathfrak{F}(\kappa')^{3/2-\alpha}\kappa'^{1/2-\beta} d\kappa' - \mathfrak{F}^{3/2-\alpha}\kappa^{1/2-\beta} \int_{\kappa}^{\infty} \mathfrak{F}(\kappa')^{\alpha}\kappa'^{\beta} d\kappa' \right] - 2\nu\kappa^2\mathfrak{F}. \quad (12)$$

Obviously if $\mathfrak{F}(\kappa)$ is known for $t = 0$, equation (12) determines the values of $\mathfrak{F}$ for all times. If one neglects the first term on the left side, which represents the decay of turbulence and chooses the specific values $\alpha = 1/2$, $\beta = -3/2$ one arrives to the theory proposed by Heisenberg.

Let us consider the case of large Reynolds number but assume that $\kappa$ is not so large that the term containing the viscosity coefficient becomes significant. Let us also assume that the first term on the left side is small by comparison to the second term. Physically this means that the energy entering in the interval $d\kappa$ is equal to the energy which leaves the interval. Then one has the relation:

$$\mathfrak{F}^{\alpha}\kappa^{\beta} \int_0^{\kappa} \mathfrak{F}(\kappa')^{3/2-\alpha}\kappa'^{1/2-\beta} d\kappa' = \mathfrak{F}^{3/2-\alpha}\kappa^{1/2-\beta} \int_{\kappa}^{\infty} \mathfrak{F}(\kappa')^{\alpha}\kappa'^{\beta} d\kappa'. \quad (13)$$

This equation is satisfied by the solution $\mathfrak{F}(\kappa) \sim \kappa^{-5/3}$, as one easily can see by substitution in 13. This result is independent, evidently, of the special choice of $\alpha$ and $\beta$. That is the reason why it was independently found by Onsager, Kolmogoroff and Weizsäcker. It is essentially a consequence of dimensional considerations. Let us now stay with the case of large Reynolds numbers by neglecting again the viscosity term but retaining the first term on the left side. In other words we consider the actual process of decay at large Reynolds numbers. Let us assume that $\mathfrak{F}$ is a function of a non-dimensional variable $\kappa/\kappa_0$, when $\kappa_0$ is a function of time. This assumption is equivalent to our former assumption that $f(r)$ is a function of $r/L$; i.e., we assume that $\mathfrak{F}$ and $f$ preserve their shapes during the decay. Evidently $\kappa_0 \sim 1/L$. Then the function $\mathfrak{F}$ can be written in the form

$$\mathfrak{F}(\kappa) = \frac{\overline{u^2}}{\kappa_0} \Phi\left(\frac{\kappa}{\kappa_0}\right).$$

Then with $\frac{\kappa}{\kappa_0} = \xi$ and

$$\frac{\partial}{\partial t}\mathfrak{F} = \frac{\Phi}{\kappa_0}\frac{d\overline{u^2}}{dt} - \frac{\overline{u^2}}{\kappa_0^2}\Phi\frac{d\kappa_0}{dt} - \frac{\overline{u^2}}{\kappa_0^2}\Phi'(\xi)\xi\frac{d\kappa_0}{dt}$$

equation (12) becomes

$$\left(\frac{1}{\kappa_0}\frac{d\overline{u^2}}{dt} - \frac{\overline{u^2}}{\kappa_0^2}\frac{d\kappa_0}{dt}\right)\Phi - \frac{\overline{u^2}}{\kappa_0^2}\frac{d\kappa_0}{dt}\Phi'\xi + \mathfrak{W}_\kappa = 0, \qquad (14)$$

where

$$\mathfrak{W}_\kappa = -C[\overline{u^2}]^{3/2}[\Phi^\alpha\xi^\beta\int_0^\xi\Phi(\xi')^{3/2-\alpha}\xi'^{1/2-\beta}d\xi' - \Phi^{3/2-\alpha}\xi^{1/2-\beta}\int_0^\infty\Phi(\xi')^\alpha\xi'^\beta d\xi'].$$

According to Loitziansky's results[1] $\frac{1}{u^2}\frac{d\overline{u^2}}{dt} = \frac{5}{\kappa_0}\frac{d\kappa_0}{dt}$ and one obtains the equation

$$\xi^5(\xi^{-4}\Phi)' = -5C\frac{[\overline{u^2}]^{3/2}}{\frac{d\overline{u^2}}{dt}}[\Phi^\alpha\xi^\beta I_0^\xi - \Phi^{3/2-\alpha}\xi^{1/2-\beta}I_\xi^\infty], \qquad (15)$$

where

$$I_0^\xi = \int_0^\xi\Phi(\xi')^{3/2-\alpha}\xi'^{1/2-\beta}d\xi'; \qquad I_\xi^\infty = \int_\xi^\infty\Phi(\xi')^\alpha\xi'^\beta d\xi'.$$

Let us assume that $4\alpha + \beta < 5/2$ as, for example, in the case of Heisenberg. Then for small values of $\xi$ the right side of equation (15) is small in comparison with the term on the left side and one has

$$\Phi(\xi) \cong \text{const. } \xi^4.$$

If $4\alpha + \beta > 5/2$, $\mathfrak{F}$ begins with a lower power of $\kappa$ than $\kappa^4$ and one can show that the integral $\int_0^\infty r^4 f(r)dr$ does not converge, so that Loitziansky's result is incorrect. I should like to investigate this second case in a later work. Let us assume, for the time being, that Loitziansky's result is correct and therefore the first case prevails. Then it follows that $\mathfrak{F}$ or $\Phi$ behaves as $(\kappa/\kappa_0)^4$ for small values of $\kappa$ and is proportional to $(\kappa/\kappa_0)^{-1/3}$ for large values of $\kappa$. For any definite choice of $\alpha$ and $\beta$ the differential equation (15) can be solved numerically. In June, 1947, I suggested to F. E. Marble that he carry out some such calculations and his results will be reported in a following publication. The result that $\mathfrak{F} \cong \kappa^4$ for small values of $\kappa$ was also found in a different way by C. C. Lin.

For the time being I propose an interpolation formula as follows:

$$\Phi(\xi) = \text{const.}\frac{\xi^4}{(1+\xi^2)^{17/6}}. \qquad (16)$$

This interpolation formula represents correctly $\Phi(\xi)$ for small and large values of $\xi$ and has the advantage that all calculations can be carried out analytically by use of known functions. The results are as follows:

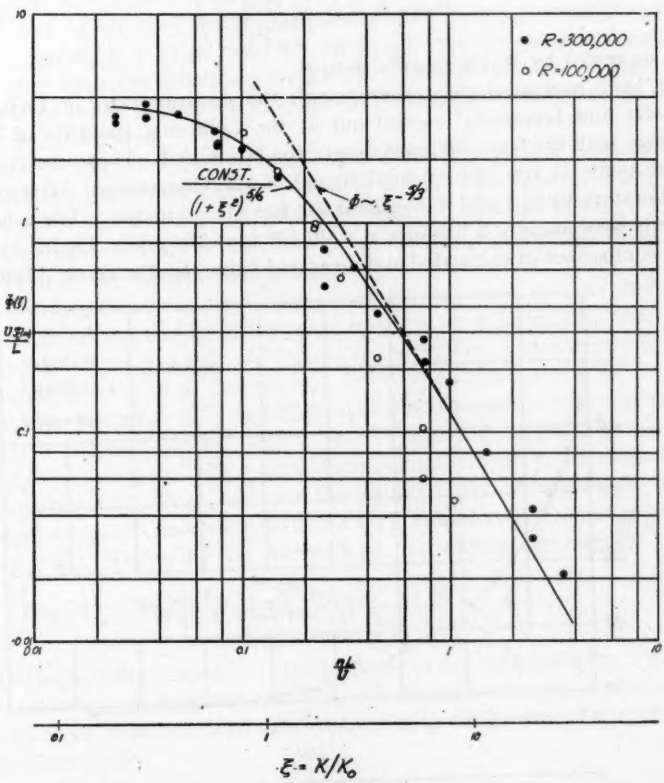


FIGURE 2

Comparison of observed and computed values of the frequency spectrum.

$$\mathfrak{F}(\kappa/\kappa_0) = \text{const.}\ \frac{(\kappa/\kappa_0)^4}{[1 + (\kappa/\kappa_0)^2]^{5/6}}$$

$$\mathfrak{F}_1(\kappa_1/\kappa_0) = \text{const.}\ \frac{1}{[1 + (\kappa_1/\kappa_0)^2]^{5/6}}$$

$$f(\kappa_0 r) = \frac{2^{5/6}}{\Gamma(1/3)} (\kappa_0 r)^{1/3} K_{1/3}(\kappa_0 r) \tag{17}$$

$$g(\kappa_0 r) = \frac{2^{2/3}}{\Gamma(1/3)} (\kappa_0 r)^{1/3} \left[ K_{1/3}(\kappa_0 r) - \frac{\kappa_0 r}{2} K_{-2/3}(\kappa_0 r) \right].$$

The $K$'s are Bessel functions with imaginary argument. For small values of $\kappa_0 r$

$$f(\kappa_0 r) = 1 - \frac{\Gamma(^2/_3)}{\Gamma(^4/_3)}\left(\frac{\kappa_0 r}{2}\right)^{^2/_3} \qquad (18)$$

as suggested by Kolmogoroff's theory.

I have compared these results with the measurements of Liepmann Laufer and Liepmann[2] carried out at the California Institute of Technology with the financial assistance of the N.A.C.A.† These observations were made in the 10-foot wind tunnel of the Guggenheim Aeronautical Laboratory using a grid whose mesh size was $M = 4$ inches. The measurements were made at a distance $x = 40.4M$ from the grid. Figure 2 shows the comparison of calculated and measured values for the spectral function

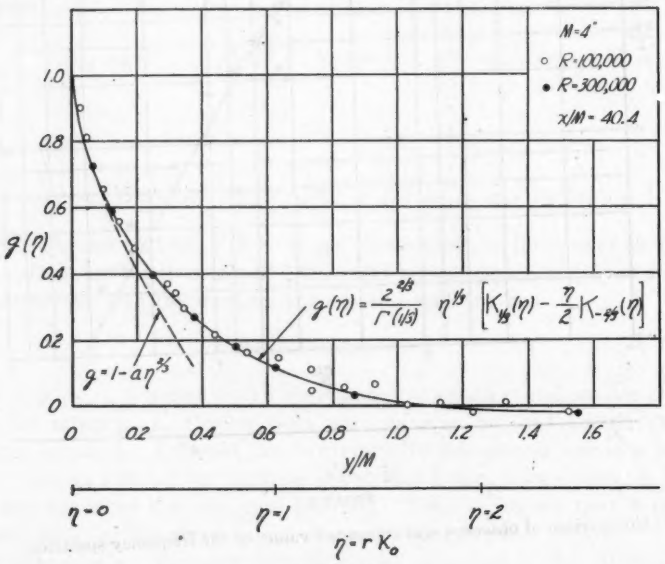


FIGURE 3

Comparison of observed and computed values of the correlation function $g$. The Reynolds number is based on the stream velocity and the mesh size.

$\mathfrak{F}_1(\kappa_1)$. It has to be taken into account that the observed values of $\mathfrak{F}_1(\kappa_1)$ have large scatter; the deviation for high values of $\kappa_1$ corresponds to the beginning influence of viscosity. Figure 3 gives a comparison between measured and calculated values of the correlation function $g(r)$. This function is chosen because the observations are more accurate than in any other case. It is seen that the agreement is almost too good in view of the assumptions made above. One must remark that there is only one arbitrary constant in the formula for $g$, viz., the constant $\kappa_0$ which deter-

mines the scale of the turbulence. It is true that some of the data of reference 2 do not show such a good agreement. The agreement is excellent for values of $g$ larger than 0.1, but after that the measured values are higher than the calculated ones. Possibly some oscillations existing in the wind tunnel stream were interpreted as turbulence or the turbulence is not quite isotropic.

I believe that the merits of my deduction are: (*a*) the assumptions involved are exactly formulated; (*b*) the specific assumptions of Heisenberg's theory concerning the transition function are not used; (*c*) the actual process of decay is considered; (*d*) the analysis is extended to the lower end of the turbulence spectrum.

Concerning the case of large values of $\kappa$ (small values of $r$) L. Kovásznay[3] introduced an interesting assumption which is more restricting than my assumption $D$. Obviously $\int_0^\kappa \mathfrak{W}_\kappa d\kappa$ is the total energy transferred by the Reynolds stresses from the interval $(0 \to \kappa)$ to the interval $(\kappa \to \infty)$. Kovásznay assumes—following Kolmogoroff's arguments—that this quantity is a function of $\mathfrak{F}(\kappa)$ and $\kappa$ only. Then for dimensional reasons $\int_0^\kappa \mathfrak{W}_\kappa d\kappa = \text{const. } \mathfrak{F}^{3/2}\kappa^{5/2}$. This assumption appears to be correct for large values of $\kappa$. When, however, the assumption is extended to the range of small values of $\kappa$ and one substitutes $\mathfrak{W}_\kappa$ in equation (8) one can calculate easily $\mathfrak{F}(\kappa)$. Neglecting the viscous term, one obtains the relation

$$\mathfrak{F}(\kappa/\kappa_0) = \text{const.} \frac{(\kappa/\kappa_0)^4}{[1 + \mathfrak{F}^{1/2}(\kappa/\kappa_0)^{3/2}]^{17/2}} \qquad (19)$$

The right side of equation (19) behaves as my corresponding equation (17) for small and large values of $\kappa/\kappa_0$. It will be interesting to see how far the different transition from small to large values influences the accordance with observation.

* Presented at the Heat Transfer and Fluid Mechanics Institute, Los Angeles, California, June 23, 1948.

† The N.A.C.A. has kindly allowed presentation of these data prior to official N.A.C.A. publication.

[1] Loitziansky, L. G., "Some Basic Laws of Isotropic Turbulent Flow," Central Aero-Hydrodynamical Institute, Report No. 440, Moscow, 1939. Translated as N.A.C.A. Technical Memorandum 1079.

[2] Liepmann, H. W., Laufer, J., and Liepmann, K., "On Some Turbulence Measurements Behind Grids," Final Report N.A.C.A. Contract NAw 5442, July, 1948.

[3] Kovásznay, Leslie, S. G., "The Spectrum of Locally Isotropic Turbulence," *Phys. Rev.*, **73**, (9), 1115 (May, 1948).

# NOTE ON THE LAW OF DECAY OF ISOTROPIC TURBULENCE

By C. C. Lin

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Communicated by Theodore von Kármán, August 2, 1948

The law of decay of turbulence has been discussed by several authors by making various assumptions. Recently, Batchelor[1] made a summarizing discussion of some of these assumptions and their consequences. The present author has made a different assumption, which seems to be suggested by Kolmogoroff's concept of turbulence at high Reynolds numbers, and yet has not been covered by Batchelor's discussion. The result seems also to be in reasonably good agreement with all experimental data available. This result was originally obtained by a discussion of the spectrum, but a derivation using the correlation function itself is presented here.

According to Kolmogoroff, the double and triple correlation functions $f(r)$ and $h(r)$ for a distance $r$ apart should be of the forms

$$u'^2\{1 - f(r)\} = v^2\beta_2\left(\frac{r}{\eta}\right), \tag{1}$$

$$u'^3h(r) = v^3\beta_3\left(\frac{r}{\eta}\right), \tag{2}$$

where $\beta_2$ and $\beta_3$ are definite functions of their argument $\xi = r/\eta$ for small values of $\xi$, at high Reynolds numbers, $u'$ is the intensity of turbulence, and $\eta$ and $v$ are characteristic measures of length and velocity. Furthermore, $\eta$ and $v$ are given in terms of the rate of energy dissipation by the following relations

$$\eta = (\nu^3/\epsilon)^{1/4}, \qquad v = (\nu\epsilon)^{1/4}, \qquad \epsilon = -\frac{3}{2}\frac{du'^2}{dt}, \tag{3}$$

where $t$ is the time, and $\nu$ is the kinematic viscosity coefficient. At the various stages of the decay process, $\epsilon$ changes. Comparing the fields at various stages of decay, one sees that $\beta_2(\xi)$ and $\beta_3(\xi)$ should be definite functions of $\xi$ for small $\xi$ in the course of time, i.e., there is self-preservation of $\beta_2(\xi)$ and $\beta_3(\xi)$.

We shall now show that if one makes the assumption of self-preservation of $\beta_2$ and $\beta_3$, (3) follows as a consequence. Also, a definite law of decay is obtained which is in agreement with all experimental data so far available.

Substituting (1) and (2) into the equation of von Kármán and Howarth,

$$\frac{\partial}{\partial t}(u'^2f) + 2u'^3\left(\frac{\partial h}{\partial r} + \frac{4h}{r}\right) = 2\nu u'^2\left(\frac{\partial^2 f}{\partial r^2} + \frac{4}{r}\frac{\partial f}{\partial r}\right),$$

one has

$$\frac{du'^2}{dt} - \frac{dv^2}{dt}\beta_2(\xi) + v^2\beta_2'(\xi)\xi\frac{1}{\eta}\frac{d\eta}{dt} + \frac{2v^3}{\eta}\left\{\beta_3'(\xi) + \frac{4}{\xi}\beta_3(\xi)\right\} = \frac{2\nu v^2}{\eta^2}\left\{\beta_2''(\xi) + \frac{4}{\xi}\beta_2'(\xi)\right\}.$$

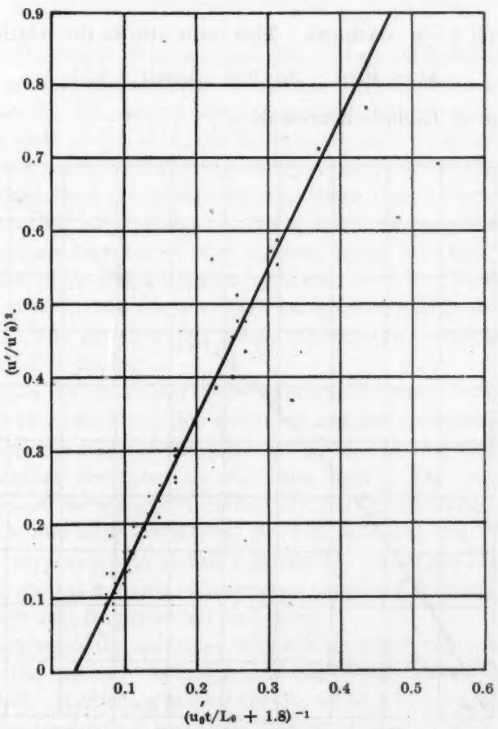


FIGURE 1

Comparison of the present law of decay with experiments.

Thus, the ratios

$$\epsilon : \frac{dv^2}{dt} : v^2\frac{1}{\eta}\frac{d\eta}{dt} : \frac{v^3}{\eta} : \nu\frac{v^2}{\eta^2}$$

must be constants. From a consideration of the first, the fourth and the fifth terms, (3) follows at once. Including the other two terms, one obtains the additional relations:

$$v^2 = A(t - t_0)^{-1}, \qquad \eta^2 = B\nu(t - t_0), \tag{4}$$

where $A$, $B$ and $t_0$ are constants. From this, it can be easily seen that

$$\epsilon = C(t - t_0)^{-2}, \tag{5}$$

and hence

$$u'^2 = \alpha(t - t_0)^{-1} + \beta, \tag{6}$$

where $C$, $\alpha$ and $\beta$ are constants. This leads also to the relation

$$\lambda^2 = 10\nu(t - t_0)\{1 + (\beta/\alpha)(t - t_0)\}, \tag{7}$$

for the change of Taylor's microscale $\lambda$.

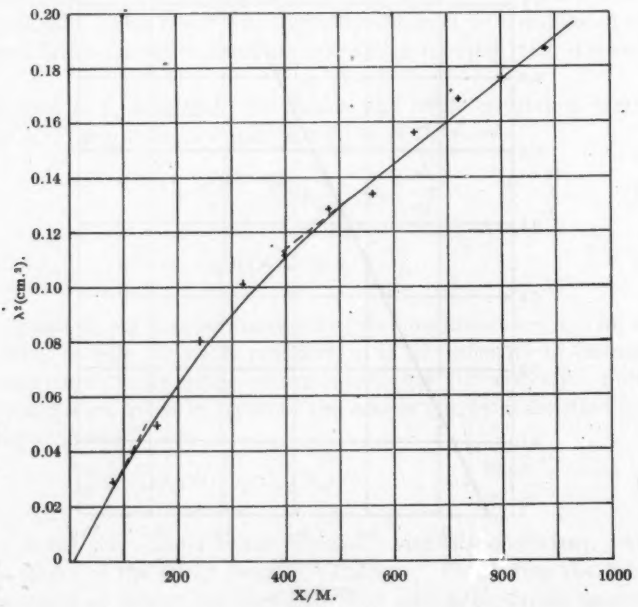


FIGURE 2

Comparison with experiments of the theoretical law of change of Taylor's microscale.

Notice that $\eta^2$ increases linearly in $t$. As $\eta$ is the smallest scale, this diffusive nature is expected. Equations (5), (6) and (7) are merely consequences of this relation. Taylor's microscale $\lambda$ does not in general possess the diffusive property any more (except when $\beta$ is zero). But it can be easily verified that $R_\lambda = u'\lambda/\nu$ and $u'^{-2}R_\lambda$ changes linearly in time.

The law of decay just obtained is different from all previous ones. It

does however include the half-power law which is at least approximately satisfied by many of the experiments. On the other hand, Dryden's analysis[2] indicates that there are certain data which do depart from it. One can however take such results into account by the present law. The comparison is shown in figure 1, where one of Dryden's diagrams (von Kármán) is replotted according to the present scheme. This is the case departing most from the half-power law. It is seen that the agreement is good.

A more critical check is to compare the theory with directly measured values of $\lambda$, as $\lambda$ depends on the derivative of $u'$. Such a comparison is made in figure 2. In making this comparison, the curve is supposed to begin with a slope of $10\nu M/U$ ($M$ being mesh size of the grid, $U = x/t$) and approaches asymptotically to a straight line of slope $4\nu M/U$. These limiting straight lines are shown dotted, where they depart considerably from the experimental values. In fact, both the experimental points and these lines are reproduced from a figure kindly supplied to the author by Mr. Batchelor, based on experimental results of Dr. Townsend. The theoretical curve is then made to join up smoothly with the asymptotic straight line. The agreement is good. It may be noted that there is no freedom in this joining.

The decay process may thus be described as follows. At the beginning of the decay process, when the Reynolds number of turbulence is high, there is self-preservation in the sense of (1) and (2) for small $r$. As the Reynolds number decreases to very low values, the self-preservation property includes the whole $f(r)$ curve. This is the final stage of the decay process which has been considered by von Kármán and Howarth and investigated very much in detail recently by Batchelor and Townsend. The physical process is much clearer when presented in terms of spectrum. This discussion will be presented elsewhere.

The appearance of the arbitrary additive constant might seem to be a drawback in the theory. Actually, it is a necessity, if one follows the idea of Kolmogoroff. According to this theory, at high Reynolds numbers, the essential decay mechanism is governed by the high-frequency components only. On the other hand, the low-frequency components do contribute to the intensity. For example, if there is a superposed disturbance of low frequencies, the essential decay mechanism should not change. The detailed discussion of this point will be presented later in connection with a study of the energy spectrum. It might be noted that when $\beta = 0$, we have the half-power law of decay, and consequently a self-preservation of $f(r)$ itself. In any case, this is approximately satisfied for the initial stages of the decay process, when $t - t_0$ is small.

[1] Batchelor, G. K., *QuAM*, 6, 97–116 (1948).
[2] Dryden, H. L., *Ibid.*, 1, 28–30 (1943).

# THE CYTOLOGICAL MECHANISM OF THE TRIPLOIDY-INDUCING EFFECT OF HEAT ON EGGS OF THE NEWT, TRITURUS VIRIDESCENS

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Communicated by E. G. Conklin, August 18, 1948

Extreme temperatures, both high and low, have been known for many years to induce polyploidy in plants. If the temperature shock is applied at the time of meiosis, diploid, and sometimes tetraploid, pollen grains are formed which, upon transfer to the styles of normal diploid plants, give rise to triploid (and, possibly, pentaploid) offspring. Treatment at a later time, during early stages of division of the zygote, may double the diploid chromosome complex and produce completely or partially tetraploid seedlings. As a rule, the percentage of polyploids resulting from temperature treatments is relatively low, ranging from less than 1% to about 13%.[1, 2, 3]

Similar experiments have been carried out with eggs of a few species of animals. Since, at the time of laying or fertilization of the eggs of most animals, the maturation divisions are not yet completed, the abnormal temperature may interfere with the completion of these divisions and thus add one or more sets of maternal chromosomes to the diploid complement of the zygote. Refrigeration of eggs of a parthenogenetic race of the brine shrimp, *Artemia salina*, in some cases caused retention of the chromosomes usually eliminated in a polar body.[4, 5] On the other hand, immersion of unfertilized eggs of the silk moth, *Bombyx mori*, in water at 46°C. for eighteen minutes resulted in the establishment of the diploid chromosome number through suppression of one of the maturation divisions. This treatment also induced the eggs to develop by parthenogenesis and fusion of diploid nuclei during cleavage produced tetraploid or partially tetraploid individuals.[6, 7] In the greenhouse white fly, *Trialeurodes vaporarorium*, virgin females parthenogenetically produce haploid males exclusively as long as the temperature remains normal. Following exposure to temperatures ranging from 37 to 45°C., virgin females gave rise to about 10% diploid female offspring, presumably through suppression of the second maturation division.[8]

Among vertebrates, the polyploidizing effect of abnormal temperatures has been investigated extensively in various species of amphibians. Refrigeration of fertilized eggs for several hours, shortly after laying, produced a high percentage of triploid larvae in the newts, *Triturus viridescens*,[9, 10] *Triturus pyrrhogaster*,[11, 12] *Triturus similans* and *rivularis*,[13] *Triton taeniatus*,[14] and *Triton alpestris*,[15, 16] and in the axolotl.[17] In some

experiments with eggs of *Triturus viridescens*, all the surviving larvae were triploid. Treatment of eggs of *Triturus viridescens* at 36 to 37°C. for five to fifty minutes gave rise to 71% triploids among the surviving embryos[18, 19] but was less effective with eggs of *Triturus pyrrhogaster*.[19,20] When eggs of the leopard frog, *Rana pipiens*, were exposed twenty minutes after insemination to 37°C. for four minutes, 38% developed into triploid embryos.[21]

As regards the mechanism responsible for the polyploidizing effect of extreme temperatures, earlier studies on plant and animal cells indicate that the change in temperature usually causes a breakdown of the spindle mechanism, either through direct action on the spindle fibers or through an effect on the centromeres or centrosomes, thus preventing the normal separation of the chromosomes at anaphase. In other cases the anaphase movements may be normal, but the two groups of chromosomes do not separate over a sufficient distance during telophase; so that a restitution nucleus is formed that incorporates all the chromosomes present in the original mitotic figure. In relatively small cells, a restitution nucleus may be formed in spite of normal telophase separation, if the abnormal temperature has no other effect than to prevent the division of the cell body.[22, 23, 24]

In the eggs of amphibians with a diameter from one to two millimeters or more, the maturation spindle is extremely small in relation to the size of the cell as a whole and located at the surface near the animal pole. On the basis of the cytological studies mentioned above, it was assumed that cold or heat would bring about a suppression of the second polar body through a breakdown of the spindle and thus lead to the formation of a diploid egg nucleus.[18, 19] This seemed to be a logical hypothesis since, at the time of laying of the amphibian egg, meiosis has progressed to metaphase of the second division but is blocked in this stage until fertilization occurs. The high percentage of triploid embryos produced by abnormal temperatures would thus be explainable by the fact that at the time of treatment the eggs always contain a mitotic figure that is arrested in metaphase, rather than an actively progressing mitosis. This reasoning was supported by the early observation that refrigeration of salamander eggs was no longer effective in inducing triploidy when the onset of the treatment was delayed until about thirty minutes after laying, at which time the second maturation division has progressed to a late anaphase.

Recently, Briggs[21] has studied the changes in susceptibility to heat treatments of eggs of *Rana pipiens* during the first forty minutes after insemination. He finds that the percentage of triploids is highest (30%) when the treatment begins twenty minutes after insemination, and drops to zero at forty minutes. At twenty minutes, or shortly thereafter, the anaphase of the second maturation division is just beginning. Possibly, the heat

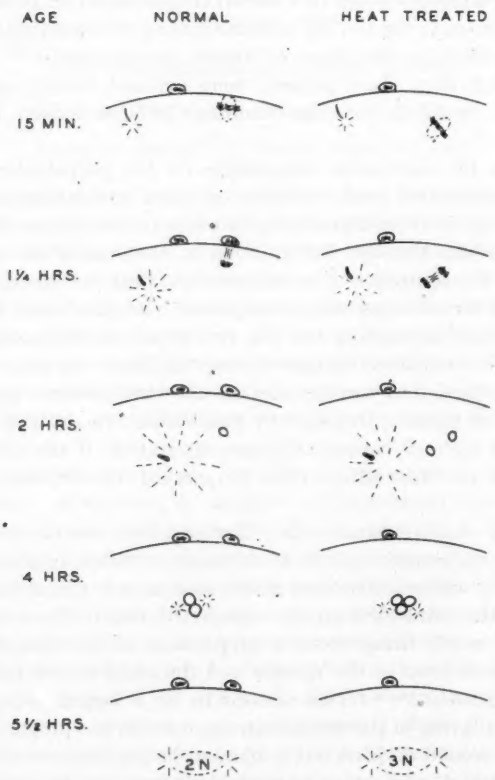


FIGURE 1

Diagrams of second maturation division and fertilization in normal eggs of *Triturus viridescens* and in eggs following exposure to 36°C. for ten minutes.

Only a small region of the egg near the animal pole is shown. Actually, the first cleavage spindle is located considerably deeper in the egg, near the center of the animal hemisphere.

Submergence of second maturation spindle in heat-treated eggs, formation of two egg nuclei, fusion of both egg nuclei with the spem nucleus, first cleavage mitosis containing the triploid number of chromosomes (thirty-three).

For details see table 1.

inhibits primarily the shortening of the chromosomal spindle fibers that may be responsible for the early anaphase separation of the chromosomes. Whatever the cytological mechanism involved, direct observation of living eggs shows that the majority of heated eggs fail to give off the second polar body, and all embryos developing from such eggs are triploid.*

In view of the importance of temperature shocks in the production of polyploid animals and the complete absence of direct observations on the mechanism involved, a cytological study of the effects of short heat treatments on eggs of the newt, *Triturus viridescens*, was begun by the junior author in the winter 1946–1947. Eggs of this species were selected for this work because they were the object of the first experiments with cold and heat on amphibian eggs, and the cytology of fertilization is known in all details.[25] Fertilization in this species, as in the great majority of salamanders, is complicated by the occurrence of "physiological" polyspermy, which may be disregarded for the purposes of this article since it does not seem to be involved in the production of triploidy by abnormal temperatures. However, the presence of supernumerary sperm nuclei, which usually degenerate in the normal course of fertilization, offers a possible explanation of the origin of various exceptional chromosomal conditions, such as haploid-diploid or haploid-triploid mosaics.

Eggs were obtained during the winter and spring by subcutaneous implantation of one or two pituitaries from female leopard frogs. The newts were allowed to deposit the eggs on sprigs of Elodea, during which process they were fertilized in normal fashion by spermatozoa present in the sperm receptacle connecting with the cloaca. The experimental eggs were removed promptly, placed for ten minutes in an incubator at 36 to 36.5°C., and transferred to a constant temperature bath running at 19.6°C. Treated eggs and controls were fixed at intervals in Bouin's or in sublimate-acetic mixture, sectioned serially at fifteen to eighteen micra, and stained with either haemalum-fast green or Feulgen-fast green.

Thus far sections of thirty-six untreated eggs fixed up to $5^1/_2$ hours from fertilization have been studied. Thirty-five show normal stages of fertilization corresponding to those described earlier;[25] one is abnormal. Among the forty-five heat-treated eggs that have been analyzed to date, twenty contain normal maturation and fertilization stages; presumably, these would have given rise to diploid embryos. Three eggs are definitely abnormal, probably unviable. The remaining twenty-two clearly show an inhibiting effect of the heat treatment on the second maturation division and illustrate the subsequent history of the mitotic mechanism in such affected eggs, as summarized in table 1 and figure 1.

It is clear from the evidence given that the most important effect of the heat treatment is not a general breakdown of the second maturation spindle but a submergence of the mitotic figure below the surface of the egg. In

TABLE 1

SECOND MATURATION DIVISION AND FERTILIZATION IN NORMAL AND HEAT-AFFECTED EGGS

| APPROXIMATE AGE (AT 19.6°C.) | NORMAL EGGS (CONTROL AND EGGS NOT AFFECTED BY HEAT TREATMENT) | EGGS SHOWING HEAT EFFECT (SUBMERGENCE OF SECOND MATURATION DIVISION)* |
|---|---|---|
| 15 mins. | Metaphase of second maturation division, spindle at right angles to and anchored at egg surface | Metaphase, spindle displaced below surface (1 egg) |
| 30 to 40 mins. | Early to late anaphase, outer pole of spindle at surface or protruding slightly | Metaphase to late anaphase, spindle at varying distance below surface, perpendicular or oblique to surface (5 eggs) |
| 1 hr. | Telophase, formation of second polar body in progress or complete, haploid set of egg chromosomes below surface | Late anaphase to early telophase, spindle at varying distance below surface, perpendicular or parallel to surface (2 eggs) |
| | | [Abortive anaphase?, far below surface, two groups of chromosomes very close together, no spindle? (1 egg)] |
| $1^1/_4$ to $1^1/_2$ hrs. | Telophase, second polar body given off, early stages in formation of egg nucleus | Telophase, spindle below surface (2 eggs) |
| | | [Abortive late anaphase or early telophase far below surface, two groups of chromosomes very close together (1 egg)] |
| $1^3/_4$ hrs. | | [Single nucleus below surface, considerably larger than in controls at 2 hrs., restitution nucleus? (1 egg)] |
| 2 hrs. | Small egg nucleus, still close to surface | Telophase, well below surface (1 egg); two small egg nuclei, identical in size and structure, close to surface, or at various distances below, not associated with any aster (4 eggs) |
| $3^1/_2$ to $4^1/_2$ hrs. | Egg and sperm nucleus in contact, in various stages of early prophase, sperm aster divided | Three nuclei (two egg nuclei and one sperm nucleus) in contact, early prophase, sperm aster divided (3 eggs) |
| $5^1/_2$ hrs. | Metaphase to anaphase of first cleavage mitosis | Late anaphase of first cleavage mitosis with 33 chromosomes at each pole (1 egg) |

* Brackets mark three cases in which submergence of the spindle may be accompanied by failure of the spindle mechanism.

untreated eggs the spindle is perpendicular to the surface, and its outer pole is anchored in the cortex. During late anaphase, the outer pole begins to protrude slightly. At one hour, formation of the second polar body is in progress or completed. In heat-affected eggs, on the other hand, the spindle is displaced below the surface and usually rotated into an oblique

position. In the majority of cases, the separation of the two haploid sets of chromosomes at anaphase appears to be normal; and, during telophase, two small egg nuclei are formed. These are spherical and completely surrounded by yolk granules and thus easily distinguished from sperm nuclei in the same egg, since the latter are still elongate or club-shaped and always located near the center of a conspicuous sperm aster.

Three eggs, fixed between $3^1/_2$ and $4^1/_2$ hours from fertilization, show the crucial stage in the formation of the triploid chromosome complement, viz., the meeting of both egg nuclei with the principal sperm nucleus in the center of the original sperm aster. The three nuclei are associated with two small asters, resulting from the division of the sperm aster, which will occupy the poles of the first cleavage spindle as in normal diploid eggs. All nuclei are identical in appearance and show an early stage of prophase. There is no doubt that such a configuration will give rise to a normal, bipolar cleavage mitosis containing the triploid number of chromosomes. This was demonstrated directly by one egg fixed $5^1/_2$ hours from fertilization which showed a normal late anaphase of the first cleavage mitosis. Exactly thirty-three chromosomes, the triploid number, were countable at one of the two poles; however, the chromosome group at the other pole was too much disturbed by the sectioning process to allow an accurate count.

Three of the twenty-two heat-affected eggs (enclosed by brackets in table 1) indicate that the treatment, in addition to displacing the second maturation spindle from the surface, may also interfere occasionally with the separation of the chromosomes at anaphase through an effect on the spindle. In two eggs there is present an "abortive" anaphase, far below the surface, without a well-defined spindle and with the two groups of chromosomes practically in contact. On one of these eggs, the condition of the chromosomes suggests the beginning of telophase so that ultimately the formation of a single, diploid nucleus would seem to be unavoidable. In the third egg, a single egg nucleus is visible, which is considerably larger than the egg nucleus in untreated eggs, and possibly represents a diploid restitution nucleus resulting from an abortive anaphase. A single polar body is present in this series of sections; however, this cannot be taken as conclusive evidence of the suppression of the second maturation division since, in normal eggs, one or both polar bodies are frequently displaced or lost during embedding and sectioning.

A change in the position of the maturation spindle under the influence of abnormal temperature has been described previously by Gross[6] in refrigerated eggs of Artemia in which the spindle may assume a position oblique or parallel to the surface. Submergence of one or both maturation divisions also occurs in unfertilized eggs of the worm Urechis following activation with either dilute sea water or ammoniacal sea water and,

probably, also after treatment with heat.[26, 27, 28] A variety of cytological conditions have been described in such eggs which, normally, do not progress beyond prophase of the first maturation division without fertilization. If both maturation divisions are submerged, four haploid nuclei are present in the egg. If the first polar body is suppressed, one of the second maturation spindles may be located at the surface and produce a normal second polar body, leaving three nuclei, i.e., a triploid chromosome complement, within the egg. However, production of polyploid embryos from such eggs is prevented, as a rule, by the appearance of an aster with each egg nucleus, leading to multipolar mitosis and irregular distribution of chromosomes. In heat-treated, fertilized salamander eggs such irregularities of the first cleavage mitosis are avoided by the fact that the division center is brought in by the sperm, as in normal fertilization.

The cytological investigations on heated eggs of *Triturus viridescens* made so far show that the primary effect of the temperature shock is a severing of the outer pole of the second maturation spindle from its anchorage in the egg cortex and a submergence of the whole spindle below the surface. The polyploidizing effect of heat may thus be connected with a change in the properties of the surface film. A study of eggs fixed during the heat treatment may show whether there is also a temporary reversible effect on the spindle fibers themselves.

The observation that in some eggs submergence of the division figure is accompanied by a permanent modification of the spindle indicates a considerable variability in the response to heat treatment by the eggs of one species and suggests that eggs of other species may give a different cytological reaction. In *Rana pipiens*, for instance, inhibition of the spindle mechanism might well be the predominant feature either with or without submergence of the mitotic figure. Also, it should be pointed out that the cytological effects of prolonged refrigeration are not necessarily the same in all details as those of short heat treatments; although the ultimate consequences as regards the chromosome number of the embryos developing from such eggs are identical.

*Summary.*—Exposure of freshly fertilized eggs of the newt, *Triturus viridescens*, to 36°C. for ten minutes induces triploidy in over 50% of the surviving embryos. Cytological study of forty-five treated eggs fixed during the first five and one-half hours after treatment confirms the hypothesis that this is accomplished through suppression of the second polar body formation which, normally, is not completed until about one hour following fertilization. Twenty-two eggs give evidence of a submergence of the second maturation spindle below the egg surface. In the majority of these eggs, the separation of the chromosomes during anaphase and telophase proceeds normally, resulting in the formation of two haploid egg nuclei. Later on, both egg nuclei unite with the sperm nucleus while

the sperm aster divides in normal fashion to furnish the poles for the first cleavage spindle. During anaphase of the first cleavage mitosis, thirty-three chromosomes, the triploid number, may be counted at the poles of the division figure.

The principal effect of the heat treatment thus appears to be dislocation of the second maturation spindle from the egg surface, rather than an inhibition of the anaphase movements of the chromosomes leading to the formation of a diploid restitution nucleus, as had previously been postulated. However, observations of three of the twenty-two eggs indicate that failure of the spindle mechanism may be superimposed on the submergence of the mitotic figure.

* A reprint has just been received of a paper by Makino and Ozima (*Cytologia*, **13**, 55–60, 1943) in which they report the suppression of the second maturation division of fertilized eggs of the carp (*Cyprius carpio*) following refrigeration. The division was arrested in anaphase and a diploid egg nucleus resulted.

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